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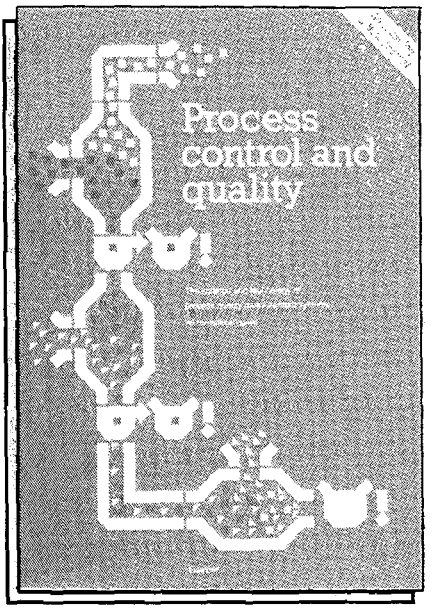

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Chromatography and Modification of Nucleosides

edited by **C.W. Gehrke** *and* **K.C.T. Kuo**, *Department of Biochemistry, University of Missouri-Columbia, and Cancer Research Center, P.O. Box 1268, Columbia, MO, U.S.A.*

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Monosized polymer particles in size-exclusion chromatography

I. Toluene as solvent

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(First received December 18th, 1989; revised manuscript received March 15th, 1990)

ABSTRACT

Monosized macroporous poly(styrene–divinylbenzene) particles were prepared by a multi-step swelling process. The preparation of particles of different sizes (5, 10 and 20 μm) with similar pore-size distributions allowed a selective evaluation of the effect of particle size on column efficiency. All three particle sizes yielded columns with similar calibration graphs, which was to be expected because of their nearly identical pore-size distributions. The uniform packing which may be achieved with monosized particles resulted in columns with high efficiency and separation capacity. With 5-μm particles more than 50 000 theoretical plates were obtained in a 30-cm column (HETP $\approx$ 0.006 mm). The resolution, as measured by the parameter R_{sp}, was good throughout the experiments and especially high for the 5-μm particles. The theoretical prediction that HETP is proportional to the square of the particle diameter was confirmed by the experimental data.

INTRODUCTION

It is well known that flow-rate and particle size in addition to packing methodology are important for column efficiency. For a certain column the main rule is that smaller particles give higher efficiency, but at the same time the resistance to flow increases, resulting in a higher pressure drop. For various applications suitable compromises between these factors must be found.

The first gel particles designed for size-exclusion chromatography (SEC) were made by cross-linking of the polysaccharide dextran (Sephadex)[1,2]. Later, gels for the separation of higher molecular weight substances were produced from agar[3].

The introduction of gel particles that swell in organic solvents[4] and later me-

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chanically stable, porous particles of poly(styrene–divinylbenzene) (SDVB)[5] offered the possibility of characterizing polymers in organic solvents. The appearance of much smaller particles of the same type provided a substantial increase in column efficiency, which allowed shorter times for an experiment[6].

As peak broadening due to mobile phase dispersion is reduced in columns packed with spherical particles having a narrow size distribution[7], it is obvious that totally monosized particles would be the best choice. Such particles could previously only be approached by repeated mechanical screening. Using the principle of activated multi-step swelling developed by Ugelstad and co-workers[8–12], it is possible to prepare highly monosized polymer particles with predetermined diameters in the range 1–100 μm. Ion-exchange materials prepared by this method have proved to be extremely efficient in protein separation processes[9].

This paper reports SEC using monosized, porous poly(styrene–divinylbenzene) particles. The separation efficiency and the flow properties were investigated using narrow polystyrene standards and *o*-dichlorobenzene. The monodispersity of the dry particles was examined by scanning electron microscopy (SEM) and their porous structure by mercury porosimetry and the BET method.

EXPERIMENTAL

Monosized particles

The chromatographic gel materials designed for this investigation were monosized porous SDVB particles[8–10], made by a two-step microsuspension process, whereby radical bulk polymerizations take place in separate micrometre-sized droplets. In the first step, a low-molecular-weight, water-insoluble compound was introduced into highly monosized 1.0-μm polystyrene seed particles. In the second step the monomer mixture was absorbed in the 'activated' seed particles and polymerized. By introduction of a non-polymerizable diluent in the monomer mixture, the particles were given a macroporous structure.

The polymer particles described were prepared with 50% DVB and with heptane as diluent in an amount of 50 vol.-% of the organic phase. After polymerization, the porogenic agent was removed by repeated washing with isopropanol followed by Soxhlet extraction with methanol. Three batches of particles were prepared with diameters of 5, 10 and 20 μm.

Specific surface areas of the particles were determined by the BET method. Pore volumes and pore radius distributions were measured by mercury porosimetry using a Carlo Erba Model 1500 instrument. A Jeol JSM 840 scanning electron microscope was used to examine the bead structure and the monodispersity of the beads.

Column packing

The gel particles were dispersed and swollen in toluene and traces of fines were removed by decanting. Steel tubes (30 cm × 7.8 mm I.D.) with 2-μm frits at the inlets and the outlets were used as columns.

Packing was performed in the following way: the column was partially filled with toluene and connected to an extension tube, then the column and the extension tube were filled with a particle slurry. After connecting a pump to the top of the extension tube, solvent was forced through the system at *ca.* 1.0 ml/min until the packing was finished. The pressure increased throughout the packing procedure.

SEC

Standard Waters Assoc. 150C SEC equipment was used for the SEC measurements. Three pulse dampers were located just after the pump in the flow system. Otherwise the equipment was in the original mode with a refractive index detector. Toluene was used as the solvent and the whole system was held at room temperature. The flow-rate was 1.0 ml/min throughout.

Calibration was performed with Waters Assoc. narrow polystyrene standards covering molecular mass ranges from $2.7 \cdot 10^6$ down to 450 g/mol. All standards were dissolved in toluene 2 h before injection. No filtration was needed. The injection volumes varied between 5 and 25 μl.

Calculations

Using efficient columns producing Gaussian peaks, the "tangent method"[13] was used to study the band broadening phenomena. The equation used was $N = 16(V_R/w)^2$, where N is the number of theoretical plates and V_R is the retention volume of the peak. The tangents to the inflection points of the elution curve were drawn from the peak to the baseline, where the difference w of the intersections was measured.

The height equivalent to a theoretical plate (HETP) was calculated as L/N, where L is the length of the column. For the calculation of N, the retention volume, V_R, and baseline peak width, w, were measured as positions on the recorder paper and then recalculated as volume. The totally monodisperse peak used was that of the solvent *o*-dichlorobenzene (ODCB).

The resolution parameter, R_{sp}, was calculated according to the definition[6,14] $R_{sp} = 0.58/\sigma D_2$, where D_2 is the slope of the calibration graph and σ is the peak standard deviation. In the calculations the average σ values of the polystyrene standards within the linear part of the calibration graphs were used. D_2 was also taken from this part of the calibration graph.

RESULTS

Monosized particles

Scanning electron micrographs of the particles demonstrating their high degree of monodispersity are shown in Fig. 1. The BET surface areas and the pore volumes are given in Table I. As shown in Fig. 2, the pore-size distributions are nearly identical for the three particle sizes. The particles contain a wide range of pore sizes, with a maximum at 1800 Å. The separation of the curves at the upper levels is due to the inter-particle void volume, which is directly related to the particle size. The steepness of the curves illustrates the high degree of monodispersity of these materials.

It should be kept in mind that BET, SEM and mercury porosimetry are methods that require drying of the gel materials. Changes in pore sizes by swelling of the porous particles in solvents such as toluene and tetrahydrofuran must be taken into consideration when the data obtained are applied to SEC. The volume swelling of the porous materials M-05A (5 μm), M-10A (10 μm) and M-20A (20 μm) in toluene are 15%, 9%, and 6%, respectively. The experimental data in Table I are for the gel materials in the dried state.

A scanning electron micrograph of the surface structure of the porous particles M-05A is shown in Fig. 3.

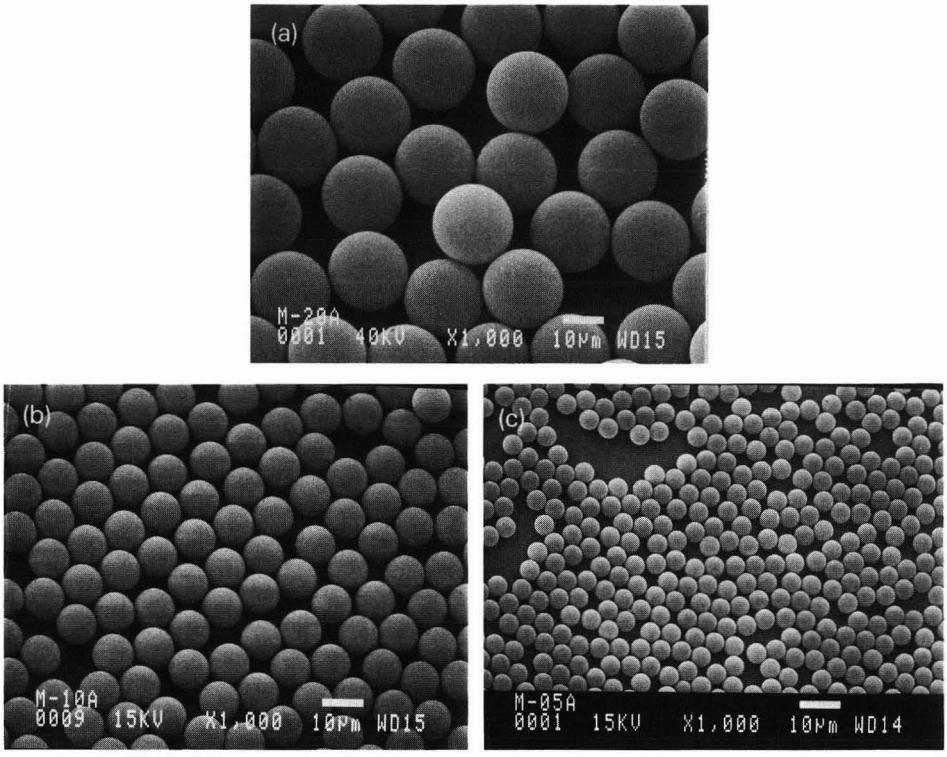

Fig. 1. Scanning electron micrographs of SDVB particles (a) 20 μm (M-20A); (b) 10 μm (M-10A); (c) 5 μm (M-05A).

Size-exclusion chromatography

For each particle size, three or more columns were packed and the performance of each column was examined. Columns producing skewed peaks were rejected, leaving three 20-μm, three 10-μm and two 5-mm columns left to characterize.

Particle diameter 20 μm. In Table II, experimental results obtained with a column packed with 20-μm particles are shown. The reproducibility of the retention volumes of both polystyrene standards and ODCB was good. The N value of ODCB was 3000 $\pm$ 200 whereas lower values were obtained for the polystyrenes. The void

TABLE I

CHARACTERISTICS OF GEL MATERIALS

Gel material	*Particle diameter (μm)*	*S_g (BET) (m^2/g)*	*Pore volume (ml/g)*
M-20A	20.6	289	1.12
M-10A	10.5	355	1.12
M-05A	5.25	261	1.04

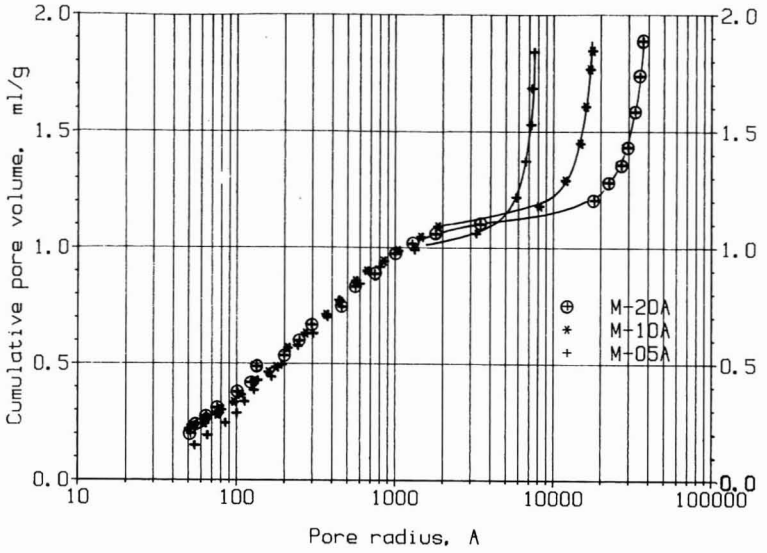

Fig. 2. Cumulative pore volume for 20-, 10- and 5-μm monosized porous particles. Copolymers with 50% porosity and 50% DVB.

volume was 6.5 ml, which corresponds to 47% of the total volume of the column. The pressure drop was 5 bar at a flow-rate of 1.0 ml/min.

The polystyrene calibration graph, showing separation in the molecular weight range $6 \cdot 10^5$–450 g/mole, is shown in Fig. 4. The graph is linear between $3 \cdot 10^5$ and $2 \cdot 10^4$ g/mol. The resolution parameter, R_{sp}, was calculated to be 2.4.

For some of the 20-μm columns, variations in the SEC conditions were applied. It was found that lower flow-rates, smaller injection volumes and higher temperatures gave higher N and R_{sp} values.

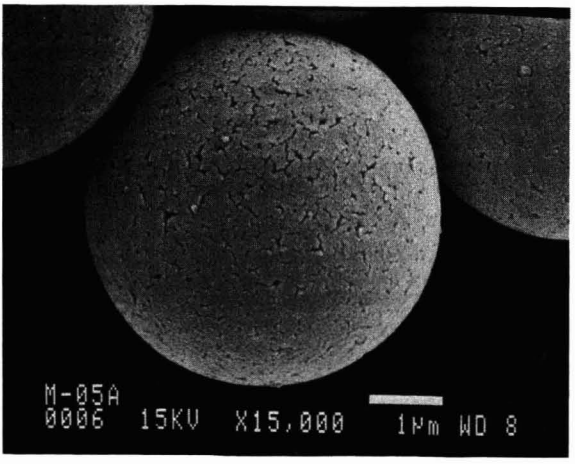

Fig. 3. Surface structure of the porous particles M-05A.

TABLE II

PERFORMANCES OF 20-, 10- AND 5-μm COLUMNS

Parameter	*Particle diameter (μm)*		
	20	*10*	*5*
Void volume (ml)	6.5	8.0	4.7
Pressure drop (bar)	5	8	65
Linear separation range (g/mol)	$3 \cdot 10^5$–$2 \cdot 10^4$	$3 \cdot 10^5$–$2 \cdot 10^4$	$3 \cdot 10^5$–$2 \cdot 10^4$
N	3000	13 000	> 50 000
HETP (mm)	0.1	0.023	< 0.006
R_{sp}	2.4	2.8	4.4

Particle diameter 10 μm. The average results are presented in Table II. An N value of 13 000 ± 3000 was found, which corresponds to a HETP of about 0.023 mm. The calibration graph resembles that of the 20-μm particles with a linear part between $3 \cdot 10^5$ and $2 \cdot 10^4$ g/mol. An average R_{sp} value of 2.8 was found. The pressure drop was 8 bar at a flow-rate of 1.0 ml/min.

Particle diameter 5 μm. The two columns yielded N values of 66 000 and 33 000. Taking N = 50 000 as an average value, the HETP becomes 0.006 mm (Table II). An average R_{sp} value of 4.4 was obtained. From the elution curve in Fig. 5, it is observed

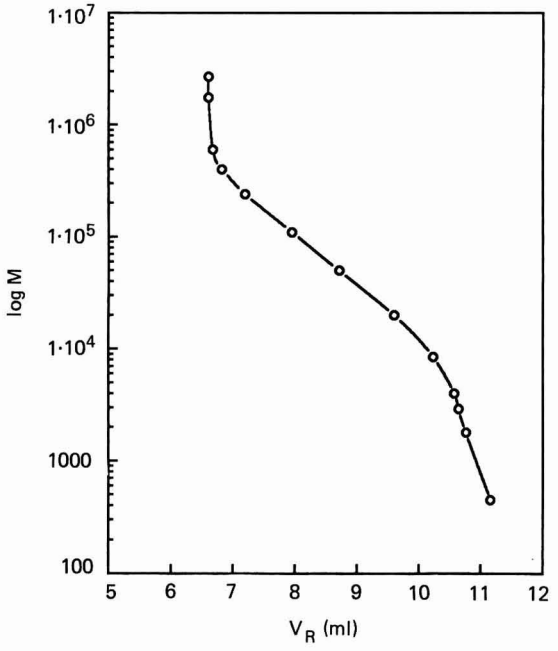

Fig. 4. PS calibration, 20-μm column.

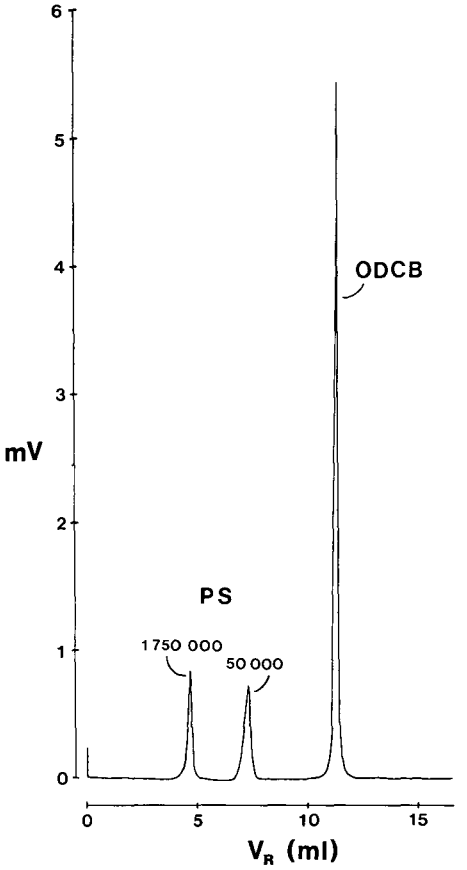

Fig. 5. Example of chromatogram obtained with PS standards (MW = $1.7 \cdot 10^6$ and $5 \cdot 10^4$) and ODCB, 5-μm column.

that the peaks of the PS standards became narrower the higher the molecular weight. The calibration graph was very similar to that for the other particle sizes. The pressure drop was as high as 65 ± 5 bar and it remained at this level throughout all the experiments. The average void volume was 4.7 ml or 35% of the total volume.

DISCUSSION

With a column I.D. of 7.8 mm and a particle material height of 290 mm, the total particle volume in a column is 13.9 ml. It is well known that a close packing of equally sized spheres occupies 74.0% of the total volume. Accordingly, the void volume of a totally close-packed column will be 3.6 ml. In practice, such a low void volume is not obtained. More realistic void volumes will be 35–40% of the total volume, *i.e.*, in the present instance 4.5–5.5 ml for a well packed column.

It should be noted that the 5-μm particle columns had a small void volume of 4.7 ml when compared with those of the columns packed with larger particles. The

TABLE III

PARTICLE DIAMETER *VERSUS* HETP

d_p (μm)	d_p^2	HETP (μm)
20.6	424	100
10.5	110	23
5.25	27.5	6

high pressure drop of the 5-μm columns could be expected to cause compression of the particles. In such a case an extra and unwanted mobile phase volume would be visible at the top of the packed bed, causing dilution of the samples and band broadening. However, no broadening behaviour was observed, and no permanent compression of the total gel volume was observed when the columns were dismantled.

All three particle sizes yielded columns with similar calibration graphs, as expected owing to the nearly identical pore-size distributions (Fig. 2).

The band broadening decreased substantially with smaller particles. It also decreased, but to a smaller extent, when the flow-rate was decreased or when the temperature was increased. With 5-μm particles more than 50 000 theoretical plates were obtained, corresponding to an HETP of the same order of magnitude as the particle diameter.

For the PS standards, band broadening increased with increasing molecular weight for the columns packed with 20- and 10-μm particles. For those packed with 5-μm particles, however, the bands visually became narrower with increasing molecular weight. An explanation is that the larger the molecules the more excluded they become, and thus the distances to diffuse in and out of the particles decrease. This tends to compensate for the slower diffusion due to higher molecular weights.

The separation power as measured by the resolution parameter, R_{sp}, increased on going from 20- to 10-μm particles. The 5-μm columns gave much higher R_{sp} values and would thus allow the separation of species with closer molecular weights. The advantage of using R_{sp} when characterizing a column is obvious. We recommend that both R_{sp} and N are calculated. N measures the efficiency of the column with respect to particle size, elution rate and packing. To characterize a column with respect to separation efficiency, calculation of the R_{sp} value is needed.

It has been deduced theoretically that the HETP should be proportional to the square of the particle diameter[6]. Monodisperse particles would be ideal for testing this hypothesis. In Table III it is shown that our experimental data are in accordance with the theory. These results give HETP $\propto d_p^{2.06}$. After correction for swelling in toluene, the exponent becomes 2.09. This is in good agreement with ref. 7.

In a future study, the pore-size distribution will be varied as a parameter. Monosized porous particles are ideal for this purpose, being the only test material that ensures identical bead properties.

CONCLUSIONS

Monosized porous poly(styrene–divinylbenzene) particles can be packed into

columns which are highly efficient for separation of mixtures of PS standards in toluene. The column performance improved considerably with decreasing particle size.

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CHROM. 22 490

Characterization of an adamantyl-modified silica used as a stationary phase in high-performance liquid chromatography

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ABSTRACT

An adamantyl-modified silica was packed and characterized by reversed-phase liquid chromatography. Initial studies involved a liquid chromatographic comparison of the adamantyl phase *versus* a conventional C_{18} stationary phase for a range of solutes including aromatic hydrocarbons, nitrobenzenes, phenols, anilines and pyridine derivatives. The coverage density of adamantyl groups on the silica surface is critical with regard to the retention behavior of these small molecules.

INTRODUCTION

This study follows up some work by Yang and Gilpin[1–3] and ourselves[4] in which adamantyl-modified silica was utilized as a reversed-phase high-performance liquid chromatographic packing. Yang and Gilpin[1] demonstrated reduced peak tailing for some basic solutes that interact strongly with residual silanols and attributed this improved peak symmetry to the bulky rigid structure of adamantane shielding the unreacted surface silanols from even small solutes. They also examined the selectivity of the adamantyl surface toward a National Institute of Standards and Technology (NITS) (formerly the National Bureau of Standards, Gaithersburg, MD, U.S.A.) test mixture of polycyclic aromatic hydrocarbons[2]. We have previously studied the effects of an adamantyl-modified silica stationary phase and other reversed-phase packings on the gradient elution of the protein ribonuclease A[4].

Similarly to Yang and Gilpin, in this study we compared the retention and selectivity characteristics of an adamantyl-modified silica packing with that of a C_{18} stationary phase for a range of solutes including an NIST test mixture of polycyclic

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aromatic hydrocarbons and an NIST test mixture for evaluating silanol activity. We also extended our earlier work[4] with adamantyl-modified silica and the gradient elution of ribonuclease A (RNase A) to more acidic conditions, to evaluate further the effect of the bulky (rigid, ball-like) structure of adamantane on the elution of proteins. The large adamantyl group should be less likely to penetrate the structure of a protein and disrupt its conformation during adsorption and elution than the thinner linear alkyl group of a conventional alkyl-bonded phase.

This work was undertaken in the hope of finding a stationary phase for reversed-phase high-performance liquid chromatography (HPLC) that would promote the irreversible unfolding of proteins to a lesser degree than the conventional *n*-alkyl bonded phases. Pearson *et al.*[5] have shown, however, that the recovery of large peptides on C_{18} phases is slightly better than on an adamantyl-bonded phase. Previously published results[4], on the contrary, have shown that ribonuclease refolds more rapidly when eluted on an adamantyl-bonded silica column than on an octadecyl-bonded column. These two sets of results are not contradictory, as Pearson *et al.*[5] analyzed denatured peptides whereas we studied the unfolding of ribonuclease[4]. This justifies the characterization of the retention properties of small molecules on the adamantyl phase.

EXPERIMENTAL

Chemicals

Bovine pancreatic ribonuclease A was purchased from Sigma (St. Louis, MO, U.S.A.) and used as received. RNase A samples were prepared as 10 and 20 mg/ml solutions in doubly distilled, deionized water.

All of the following chemicals were of analytical-reagent grade, HPLC-grade or better from J. T. Baker (Phillipsburg, NJ, U.S.A.) unless stated otherwise: acetone, acetonitrile, methanol, 2-propanol and carbon tetrachloride. Trifluoroacetic acid was purchased from Sigma and all of the solutes which were tested were obtained from Aldrich (Milwaukee, WI, U.S.A.).

Water was doubly distilled and deionized before use and all mobile phases were filtered using a vacuum filtration apparatus and 0.45-μm nylon 66 membrane filters (Schleicher & Schüll, Keene, NH, U.S.A.) before being degassed by helium sparging prior to use.

The stationary phase materials were Partisil-10 ODS-3 (C_{18}) from Alltech (Deerfield, IL, U.S.A.) and an adamantyl-bonded Partisil-10 silica. The adamantyl phase was prepared by adding 20 g of adamantylethyltrichlorosilane (Petrarch Systems, Bristol, PA, U.S.A.) to a round-bottomed flask containing 50 ml of freshly distilled toluene, 10 g of silica and 0.5 ml of pyridine and heating at 60°C for 14 h. The solid was then filtered, washed with 200 ml of methanol and finally dried at 100°C under vacuum. The particle size of both materials was 10 μm with average pore sizes of 85 Å.

Apparatus

Two chromatographic systems were employed. One consisted of a Series 400 solvent-delivery system, an LC-235 diode-array UV detector, a GP-100 graphics printer (Perkin-Elmer, Norwalk, CT, U.S.A.) and an electric six-port injector with

a 5-μl sample loop (Valco, Houston, TX, U.S.A.). The temperature difference between between the mobile phase and the column was minimized by inserting a coiled tube, with a volume of *ca.* 3 ml, between the pump outlet and the injector and immersing both the tubing and the column in a thermostated water-bath. The other system was an HP 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.). Columns were packed using a Haskel pump and a laboratory-made slurry reservoir.

Procedures

The HPLC columns were packed using 10 cm × 0.46 cm I.D. stainless-steel tubing, Parker stainless-steel unions and 0.5-μm stainless-steel frits from Alltech. Columns were packed under constant pressure (6000 p.s.i.) with the empty column filled with carbon tetrachloride and using a slurry solvent of cyclohexanol–2-propanol (50:50, v/v) with methanol as the push solvent.

RESULTS AND DISCUSSION

Initial comparisons between our adamantyl and the Partisil C_{18} stationary phases with the solute toluene indicated less carbon coverage on the former material, as suggested by the retention factors (k') for toluene in Table I. For obvious steric reasons, interactions between phenyl and adamantyl groups are also less favorable to retention than those between phenyl and long alkyl groups.

Table I also seems to indicate a stronger contribution to the retention from polar rather than hydrophobic interactions on the adamantyl phase, whereas the C_{18} phase seems to exhibit much stronger hydrophobic than polar interactions. The increasing hydrophobicities of phenol, 3-methylphenol, and 2-ethylphenol are in agreement with their increasing k' values on both phases, but the retentions are greater on the C_{18} phase. The retention factors also increase faster with increasing water content of the phase and with increasing size of the alkyl substituent(s) of the molecule.

Conversely, the much more polar 4-nitrophenol and the strongly basic pyridine and methylpyridines have smaller k' values on the C_{18} phase than on the adamantyl phase, illustrating the greater hydrophobic interactions of the C_{18} phase and the greater polar interactions of the adamantyl phase. We also observed a similar behavior of the k' values of 1,4- and 1,2-dinitrobenzene. Changing the polar characteristics of the compounds by changing the relative position of the substituents resulted in a greater change in their relative retentions on the adamantyl than on the C_{18} phase.

Evaluation of silanol activity

Both the adamantyl and the C_{18} phases were evaluated for silanol activity with an NIST recommended test mixture consisting of N,N-diethyl-*m*-toluamide (DETA) and anthracene. This mixture permits an evaluation of the relative column polarity resulting from accessible free silanols and therefore assists in predicting column suitability for the separation of polar compounds. The selectivity coefficient $\alpha_{DETA/anthracene}$ reflects the silanol activity.

The DETA–anthracene test uses acetonitrile as the mobile phase at a flow-rate of 1 ml/min and a temperature of 22°C provided by the water-bath. If the observed elution order is DETA followed by anthracene, this is an indication of a low silanol

TABLE I

RETENTIONS (k') ON ADAMANTANE AND C_{18} COLUMNS WITH WATER–METHANOL ELUENTS

Column	*Solute*	*Water in methanol (%)*			
		25	*50*	*75*	*100*
Adamantane	Phenol	0.17	0.67	1.67	3.61
	3-Methylphenol	0.26	0.97	3.35	
	2-Ethylphenol	0.4	1.58	6.39	
	4-Nitrophenol		0.81	2.61	6.98
	1,4-Dinitrobenzene	0.27	0.94	2.75	
	1,2-Dinitrobenzene	0.37	1.58	5.89	
	Aniline		0.67	2.0	5.48
	2-Ethylaniline	0.42	1.48	5.27	21.5
	Pyridine		1.48	5.12	30.42
	4-Methylpyridine	0.92	3.58	18.65	
	2-Methylpyridine	0.83	3.25	16.25	
	Toluene		0.34	1.75	
C_{18}	Phenol	0.23	0.81	3.65	19.2
	3-Methylphenol	0.27	1.57	9.5	
	2-Ethylphenol	0.5	3.38	24.65	
	4-Nitrophenol		0.35	1.54	14.61
	1,4-Dinitrobenzene	0.27	1.5	7.42	
	1,2-Dinitrobenzene	0.26	1.38	8.5	
	Aniline		0.5	2.2	12.69
	2-Ethylaniline	0.38	2.11	12.92	42.42
	Pyridine		0.44	1.69	13.54
	4-Methylpyridine	0.27	0.88	4.96	53.27
	2-Methylpyridine	0.15	0.81	4.11	48.3
	Toluene	1.04	8.0		

activity. The relative retention of DETA to anthracene increases with increasing silanol activity. DETA elutes last for the most active columns.

In our tests, the relative retention of DETA to anthracene was 5.08 on the adamantyl phase and 1.0 on the C_{18} phase. This illustrates the much stronger silanol activity and therefore the greater polarity of the adamantyl phase compared with those of the C_{18} phase. This greater polarity may be explained, at least in part, by the difference in the chemical nature of the bonding, and hence in the chemical nature of the surface prepared. The C_{18} phase is a monomeric alkyl phase, prepared with a monochlorodimethylsilane whereas the adamantyl phase is prepared with a trichlorosilane. Although it is improbable that the adamantyl phase could rightly be called a polymeric phase, the steric hindrance of the bulky adamantyl groups preventing any extent of polycondensation with water close to the silica surface, the density of residual silanol groups is probably more important with the adamantyl than with the C_{18} phase. The adamantyl groups, although large, seem to be unable to shield sufficiently these silanol groups and to prevent them from interacting with small-molecule probe solutes.

Evaluation of selectivity for polynuclear aromatic hydrocarbons (PAHs)

As in an earlier study[2], we examined the adamantyl phase with respect to PAH selectivity by using a test mixture of PAHs provided by the NIST. Sander and Wise[6–8] proposed and demonstrated that selected PAH solutes (*e.g.*, benzo[*a*]pyrene, BaP; phenanthro[3,4-*c*]phenanthrene, PhPh; and 1,2:3,4:5,6:7,8-tetrabenzonaphthalene, TBN) can be used to classify phases in terms of their selectivity.

Both the adamantyl and the C_{18} phases were evaluated using acetonitrile–water

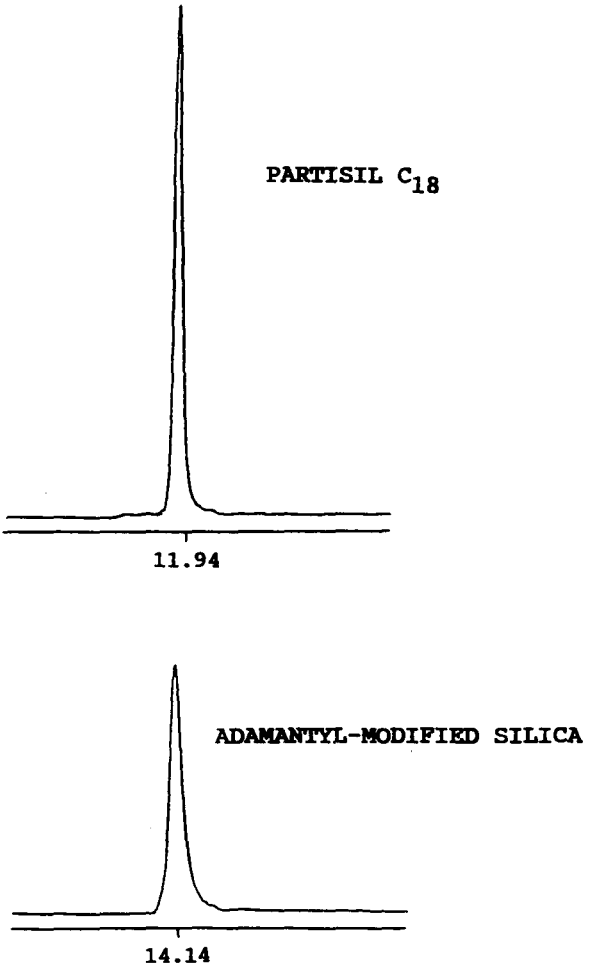

Fig. 1. Behavior of ribonuclease A on Partisil C_{18} and adamantyl-modified silica stationary phases. Eluent: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient from 0 to 85% B in 25 min, linear. Flow-rate, 1 ml/min; Sample size, 100 μg RNase A; Temperature, 20°C; Detection, 280 nm (0.2 a.u.f.s.).

(85:15, v/v) as the mobile phase at flow-rates of 1 and 2 ml/min and a temperature of 22°C. The relative retentions TBN/BaP (2.22) and PhPh/BaP (1.41) for four adamantyl phase compared favorably with those for the adamantyl phase (2.10 and 1.47, respectively) of Yang and Gilpin[2]. This, and the low surface coverage of our adamantyl phase, suggest a degree of independence of the relative retention for the PAHs studied on the surface covered by adamantyl groups.

The $\alpha_{TBN/BaP}$ value of 1.93 for our Partisil-10 ODS-3 (C_{18}) phase was identical with that found by Sander and Wise[7] for a Partisil-5 ODS-3 (C_{18}) phase. Sander and Wise classified the probable synthesis chemistry of the phases based on their experience and have found that $\alpha_{TBN/BaP}$ values greater than 1.7 and the elution order (BaP < PhPh < TBN, as observed with the adamantyl phase) characterize monomeric phases. We also found that the k' values on our adamantyl phase were about 5–6 times less than those on our Partisil C_{18} and 3–4 times less than those reported by Yang and Gilpin[2] for their adamantyl phase. These low k' values again seem to illustrate the possibility of a lower coverage of adamantane on our silica than that achieved by Yang and Gilpin, as did the fairly high polar interactions mentioned earlier.

Gradient elution of ribonuclease A

In a brief extension of an earlier study[4] we examined the effect of protein adsorption and elution on both the adamantyl and the C_{18} phases in a more acidic medium. This solvent system consisted of 0.1% trifluoroacetic acid (TFA) in water as one solvent (A) and 0.1% TFA in acetonitrile as the second solvent (B), with a gradient from 100% A to 85% B in 25 min at 25°C. This gradient was utilized because of its ability to denature completely the RNase A at 25°C, unlike the gradient system used in the previous study[4]. Fig. 1 shows the chromatograms obtained with the adamantyl and C_{18} phases for a sample size of 100 µl. It is obvious that the protein seems to be completely denatured on both columns from the sharp peak shapes in comparison with those in the previous study[4]. In agreement with what was reported previously[4], the retention time of RNase A was longer on the adamantyl than on the C_{18} phase.

CONCLUSION

The results indicate that whereas hydrophobic interactions contribute much more than polar interactions to retention on a Partisil C_{18} column, the converse is true on adamantyl phases at low surface coverage. As the earlier work of Yang and Gilpin[1] demonstrated an inhibition of polar interactions as a result of the bulky structure of adamantane blocking the underlying free silanols, it seems reasonable to assume that the polar effects and the silanol activity observed on our adamantyl phase are due to a low coverage density of adamantane on the surface of the silica. It is remarkable, however, that the presence of these polar effects does not seem to affect the PAH selectivity of our adamantyl phase compared with that of Yang and Gilpin[2].

Unlike the results of previous work[4] where we found differences between the gradient elution profile of RNase A on our adamantyl phase and other reversed-phase columns, we did not find any significant differences between the RNase A profiles on our adamantyl phase and the Partisil C_{18} phase when eluted under more acidic conditions.

Based on these results, the importance of a good coverage of adamantane on the

surface of the silica is critical with regard to the adsorption and elution of small basic molecules and the reduction of free silanol accessibility. Because of the bulkiness of the adamantyl group, a high coverage density is difficult to achieve, in agreement with the low reaction rate observed. The use of a monochlorodimethylsilane in the synthesis and the inclusion of an end-capping step in the preparation of the stationary phase would probably give a less polar surface. However, the amount of adamantane coverage did not seem to play a dominant role in the adsorption and elution RNase A. The difficulty in achieving a low density of residual silanols could explain also the previous results of Pearson *et al.*[5] and the slight loss that they observed in their study of protein recovery.

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Reversed-phase chromatographic separation of selected hydroxyl aromatics with β-cyclodextrin as a mobile phase additive

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ABSTRACT

The capacity factors and selectivity factors of several pairs of hydroxyl aromatics were obtained with a C_{18} column with and without β-cyclodextrin in methanol–water mobile phases. In all but one case, the selectivity factors were improved. The hydroxyl aromatics chosen for the study were structurally similar and difficult to separate by reversed-phase chromatography. The relationship between α values and dissociation constants of the β-cyclodextrin-solute complexes was also considered and an approximate linear relationship was found between two parameters. In general, very substantial improvements were obtained for the separation of hydroxyl aromatics with β-cyclodextrin in the mobile phase.

INTRODUCTION

The separation of structural isomers and closely related compounds is very important in chromatography. The use of cyclodextrins in liquid chromatography has been shown to be effective in the separation of several compounds[1]. Cyclodextrins are oligosaccharides containing glucose units joined by α-1,4-linkages into a cone-shaped torus. α-Cyclodextrin (α-CD) is formed by the binding of six glucopyranose units, whereas β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD) are formed by binding of seven and eight glucopyranose units, respectively. The glucopyranose units are chemically bonded such that the cavity that is formed is hydrophobic. The cavity is occupied by ether-like oxygen and C–H functionality. However, the outside of the cavity is hydrophilic due to hydroxyl groups. Molecules that have the correct sizes and shapes can fit inside of the cavity and form an inclusion complex and the inclusion complex can affect the retention of a solute in a chromatographic system. This is why cyclodextrins have been used in two different ways in high-performance liquid chromatography (HPLC), namely, either bonded to the stationary phase or dissolved in the mobile phase.

Dębowski and co-workers[1–4] reported the separation of mandelic acid into

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enantiomers, the separation of several isomers, and the separation of some aromatic amino acids by reversed-phase HPLC using α- or β-CD as a mobile phase component. Sybilska *et al.*[5] applied α- and β-CD to the separation of *o*-, *m*- and *p*-nitro-*cis*- and *trans*-cinnamic acids by reversed-phase HPLC. Gazdag *et al.*[6] reported the separation of optical isomers of D,L-norgestrel using α-, β- and γ-CD as mobile phase additives in HPLC. Gazdag *et al.*[7] applied α-, β- and γ-CD as mobile phase components in HPLC systems for the separation of isomeric estrogens in HPLC systems. Shimada *et al.*[8] discussed the use of cyclodextrins in the mobile phase for the separation of isomeric estrogens in HPLC systems. Sybilska *et al.*[9] discussed the use of α-CD for the separation of *o*-, *m*- and *p*-nitrobenzoic acids by reversed-phase HPLC. Dębowski *et al.*[10] studied the separation of isomeric alkylbenzenes in reversed-phase HPLC using aqueous mobile phases with α- and β-CD. In addition, β-CD and its derivatives have been used as selective agents for the separation of several compounds and isomers by HPLC[11–13]. Other aspects of β-CD in the mobile phase have also been considered[14].

In this work, the separation of several structurally similar compounds and structural isomers are reported that are normally very difficult to separate. Reversed-phase HPLC with methanol–water mobile phases and β-CD as a mobile phase additive, were used to separate the compounds.

EXPERIMENTAL

Apparatus

The liquid chromatograph used was a Waters unit with a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a U6K injector, a dual-channel ultraviolet detector set at 254 nm and a dual-channel 10-mV strip chart recorder. The column temperature was kept constant at 25°C by a Model FIAtron temperature controller. The column employed was a 10-μm Bondapak C_{18} (30 cm × 3.9 mm I.D.) purchased from Phenomenex.

Reagents

The model compounds were obtained from commercially available sources. The model compounds and β-CD were purchased from Aldrich (Milwaukee, WI, U.S.A.). Methanol was HPLC grade and obtained from Baker (Phillipsburg, NJ, U.S.A.).

Procedures

Methanol and water were prefiltered through a Millipore type FH 0.5 filter. β-Cyclodextrin was vacuum dried at 75–80°C for 8 h before use. Then, β-CD was dissolved in purified water and methanol was added to the β-CD solution. The model compounds were dissolved in methanol. The temperature was kept constant at 25°C and the column void volume was obtained by injecting a methanol solution of potassium nitrite.

RESULTS AND DISCUSSION

Separation of model compounds on a 10-μm C_{18} column

Previously, we investigated β-CD as a mobile-phase modifier in the reversed-

phase chromatography of polycyclic aromatic hydrocarbons, nitrogen heterocycles and hydroxyl aromatics[14]. A wide range of β-CD concentrations was employed and the retention properties of the compound classes were compared in methanol–water and ethanol–water mobile phases at 25°C. β-CD–solute dissociation constants were obtained for the compounds, and several conclusions were made about the mechanism of retention with β-CD in the mobile phase. In addition, comparisons were made between the structural features of the compounds and their capacity factors and dissociation constants. It was found that the retention properties of polycyclic aromatic hydrocarbons and nitrogen heterocycles were affected little by the presence of β-CD in the mobile because these solutes interacted more readily with the stationary phase rather than the β-CD in the mobile phase. Further, the retention characteristics of most of the hydroxyl aromatics were affected significantly with β-CD as a mobile phase modifier, indicating that β-CD could compete with the C_{18} column for the solute[14]. Because of our previous results with hydroxyl aromatics, it was decided to investigate the separation of structurally similar and isomeric hydroxyl aromatics with β-CD as a mobile phase modifier.

Fig. 1 shows the separation of 2,3-dihydroxynaphthalene and *p,p'*-biphenol in methanol–water (40:60) and in methanol–water (40:60) with 5.0 m*M* β-CD. In the absence of β-CD, the chromatographic bands of the two compounds overlapped severely and no separation was achieved. On the other hand, with β-CD in the mobile phase, the two compounds were easily separated and baseline resolution was obtained. This was due mainly to the formation of an inclusion complex between β-CD with *p,p'*-biphenol which resulted in a large decrease in the retention of *p,p'*-biphenol[14]. The separation of the model compounds can be related to their dissociation constants (K_D)

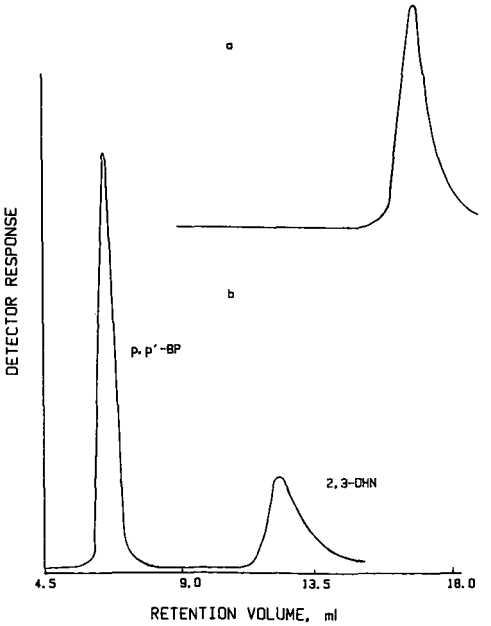

Fig. 1. Chromatograms of mixtures of 2,3-dihydroxynaphthalene (DHN) and *p,p'*-biphenol (*p,p'*-BP) in methanol–water (40:60) (a) and methanol–water (40:60) with 5.0 m*M* β-CD (b) on a 10-μm C_{18} column.

with β-CD. As discussed earlier, the smaller the K_D value, the smaller is the capacity factor (k')[14]. For instance, the K_D values of *p,p'*-biphenol and 2,3-dihydroxynaphthalene in methanol–water (40:60) with 5.0 m*M* β-CD are $4.10 \cdot 10^{-4}$ and $2.17 \cdot 10^{-3}$ *M*, respectively[14]. Thus, the decrease in k' for *p,p'*-biphenol would be larger than that for 2,3-dihydroxynaphthalene.

5-Indanol and *o,o'*-biphenol had very similar retention characteristics in methanol–water (40:60) so that the two peaks overlapped completely. With β-CD in the mobile phase, the separation of the mixture was easily achieved and baseline resolution was readily attained (Fig. 2). The K_D values for 5-indanol and *o,o'*-biphenol in methanol–water (40:60) are $1.41 \cdot 10^{-3}$ and $4.75 \cdot 10^{-3}$ M, respectively[14]. Since the K_D value of *o,o'*-biphenol is larger than that of 5-indanol, 5-indanol did not interact as readily with the stationary phase as did *o,o'*-biphenol. Other compound mixtures also showed good separations with β-CD present in methanol–water mobile phases. Some of these are shown in Figs. 3 and 4. Other compound pairs that showed excellent separation with β-CD in the mobile phase were 1,7-dihydroxynaphthalene and *p,p'*-biphenol, and 2-naphthol and *o,o'*-biphenol.

Separation of structural isomers

The separation of structural isomers in HPLC is of great importance. In this work, a mixture of 2-, 3- and 4-phenylphenol showed only two peaks in methanol–water (40:60). One peak belonged to 2-phenylphenol, and the other peak consisted of 3- and 4-phenylphenol. However, three peaks with baseline resolutions were obtained in methanol–water (40:60) containing 5 m*M* β-CD. The chromatograms are shown in Fig. 5a and b. The chromatograms indicated that the retention of 4-phenylphenol was

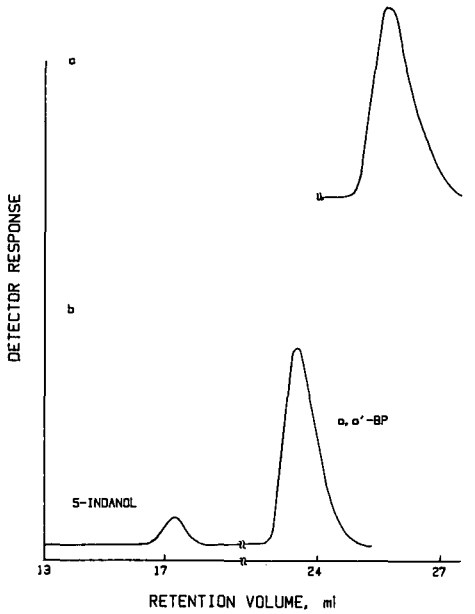

Fig. 2. Chromatograms of mixtures of 5-indanol and *o,o'*-biphenol (*o,o'*-BP) in methanol–water (40:60) (a) and methanol–water (40:60) with 5.0 m*M* β-CD (b) on a 10-μm C_{18} column.

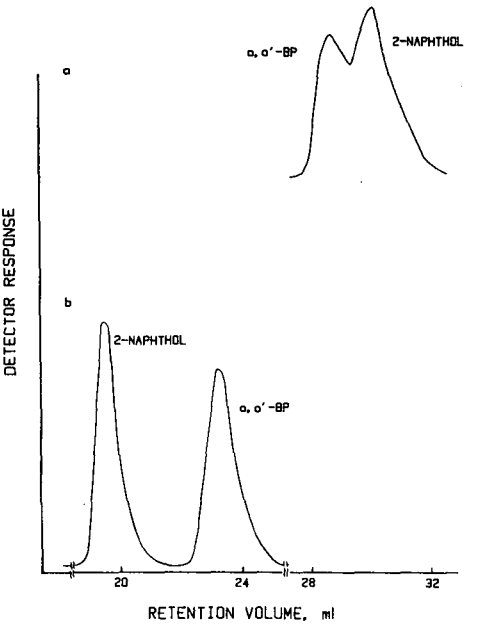

Fig. 3. Separation of *o,o'*-biphenol (*o,o'*-BP) and 2-naphthol in methanol–water (40:60) without (a) and with (b) 5.0 m*M* *β*-CD on a 10-*μ*m C_{18} column.

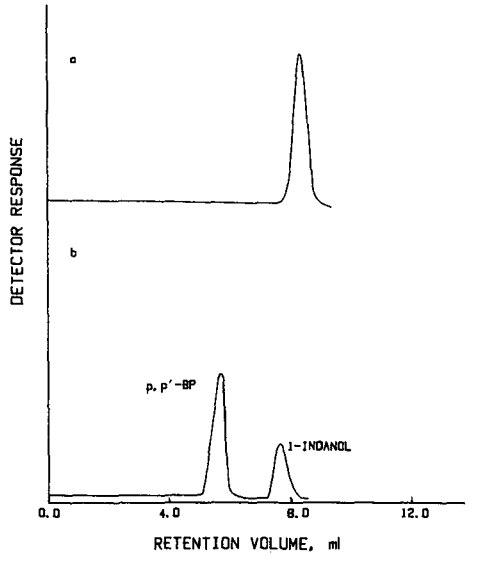

Fig. 4. Chromatograms of mixtures of *p,p'*-biphenol and 1-indanol in methanol–water (50:50) (a) and methanol–water (50:50) with 4.0 m*M* *β*-CD (b) on a 10-*μ*m C_{18} column.

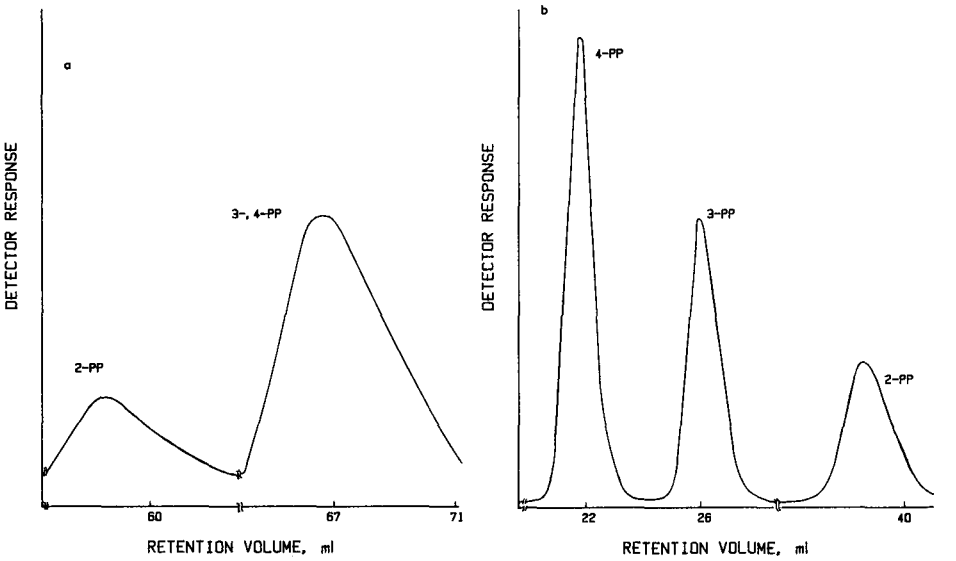

Fig. 5. Separation of phenylphenols in methanol–water (40:60) without (a) and with 5.0 m*M* β-CD (b) on a 10-μm C_{18} column.

substantially reduced and was the least retained of the isomers with β-CD in the mobile phase (Fig. 5). The separation resulted because a stronger β-CD inclusion complex formed with 4-phenylphenol than with 2- and 3-phenylphenol. The changes in k' values for 2-, 3- and 4-phenylphenol, due to the addition of β-CD, were related to their K_D values which are $1.74 \cdot 10^{-3}$, $6.18 \cdot 10^{-4}$ and $4.72 \cdot 10^{-4}$ M, respectively[14]. It can be seen that 4-phenylphenol has the smallest K_D value, and therefore it would be expected to have smaller k' values with β-CD in the mobile phase compared to the other phenylphenol isomers.

Selectivity factors

The selectivity factor (α) for the model compounds in methanol–water mixtures with β-CD can be obtained from the following equation,

$$\alpha = \frac{k'_{02}K_{D2}}{k'_{01}K_{D1}}\left(1 + \frac{K_{D1} - K_{D2}}{K_{D1} + [CD]_m}\right)$$

where k'_{01} and k'_{02} are the capacity factors of components 1 and 2 in the absence of β-CD, K_{D1} *and* K_{D2} are the dissociation constants of the inclusion complexes of components 1 and 2 with β-CD, respectively, and $[CD]_m$ is the equilibrium concentration of β-CD. The above equation is similar to the equation used by Fujimura *et al.*[15]. However, they used total β-CD concentration in the equation. We have discussed the use of the equilibrium concentration in relating k' values to K_D values with β-CD in the mobile phase[14]. Table I lists the capacity factors and selectivity factors of several pairs of compounds in different methanol–water mobile phases with β-CD on a 10-μm column. As shown in Table I, the α values increased with addition of

TABLE I

CAPACITY FACTORS AND SELECTIVITY FACTORS FOR PAIRS OF HYDROXYL AROMATICS WITH β-CYCLODEXTRIN PRESENT IN THE MOBILE PHASE

Compound pairs[a]	*Methanol–water*	*k'*	α^b
2,3-DHN	40:60	3.01	1.67
p,p'-BP	40:60	1.80	
1-Indanol	40:60	3.88	1.00
1,7-DHN	40:60	3.88	
5-Indanol	40:60	6.25	1.43
o,o'-BP	40:60	8.92	
1-Indanol	50:50	7.75	1.67
p,p'-BP	50:50	5.60	
o,o'-BP	30:70	15.82	1.50
2-naphthol	30:70	10.52	
3-PP	40:60	6.22	1.22
4-PP	40:60	5.10	
p,p'-BP	57:43	0.958	1.21
1-DHN	57:43	1.16	
1-Indanol	60:40	1.31	1.31
1,7-DHN	60:40	0.997	

[a] DHN = Dihydroxynaphthalene; BP = biphenol; PP = phenylphenol.

[b] Without β-CD in the mobile phase the α values were equal to 1.00, except for *o,o'*-BP and 2-naphthol, and 1-indanol and 1,7-DHN.

β-CD, except for one pair of compounds. As an example, no separation was achieved for the mixture of *p,p'*-biphenol and 2,3-dihydroxynaphthalene ($\alpha = 1.00$), whereas baseline resolution was attained with the addition of β-CD ($\alpha = 1.67$). As another example, the α value improved from 1.00 with no β-CD to 1.43 with 5.0 m*M* β-CD in methanol–water (40:60) for the separation of 5-indanol and *o,o'*-biphenol. The α values listed in Table I were the same for a given pair of compounds whether they were calculated with the *k'* values for the pure compounds or calculated from *k'* values obtained from a mixture of a given pair of compounds.

It is instructive to make a comparison between the change in the selectivity factors of pairs of compounds with and without β-CD and the corresponding ratio of their K_D values. Fig. 6 shows the relationship between $\Delta\alpha$ and the ratio of K_D values in methanol–water (40:60) for four pairs of compounds. The $\Delta\alpha$ value refers to the difference in the selectivity factor of a pair of components in a mixture with and without β-CD in the mobile phase. The points in Fig. 6 represent mixtures of 3- and 4-phenylphenol (point 1), 2-naphthol and *o,o'*-biphenol (point 2), 5-indanol and *o,o'*-biphenol (point 3) and *p,p'*-biphenol and 2,3-dihydroxynaphthalene (point 4). Points 1, 3 and 4 fell on a straight line. As shown in Fig. 6, point 2 did not fall near the line. The linear relationship indicated that as the K_D ratio increased, the difference in selectivity factors increased and better separation was achieved. *p,p'*-Biphenol and 2,3-dihydroxynaphthalene (point 4) were separated with a larger $\Delta\alpha$ value compared to

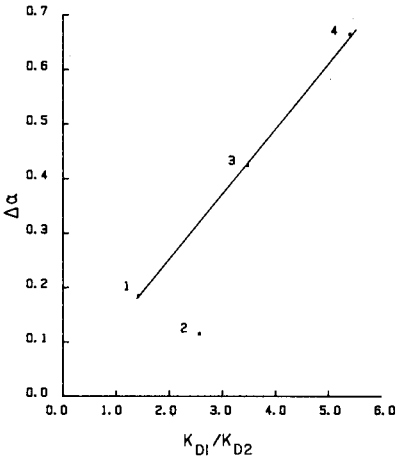

Fig. 6. Plot of $\Delta\alpha$ *vs.* the K_D ratio for several pairs of hydroxyl aromatics in methanol–water (40:60) on a 10-μm C_{18} column. See text for discussion of data points.

5-indanol and *o,o'*-biphenol and 3- and 4-phenylphenol in methanol–water (40:60). These results indicated that the separation of the model compounds was related to their ability to form inclusion complexes with the β-CD in methanol–water (40:60) and followed a similar chromatographic mechanism. However, other types of interactions were occurring for either 2-naphthol and *o,o'*-biphenol as indicated by the fact that point 2 did not fall on the line in Fig. 6.

CONCLUSIONS

The addition of β-CD to the mobile phase in the reversed-phase chromatographic system investigated resulted in dramatic improvements in the separation of structurally similar hydroxyl aromatics. Without β-CD in the mobile phase, many of the pairs of compounds could not be separated with methanol–water or were only partially separated. However, with β-CD the compound pairs were easily separated. With the phenylphenol isomers, only two chromatographic bands were obtained with methanol–water. However, by the addition of β-CD to the mobile phase, the phenylphenol isomers were readily separated and baseline resolution was obtained for the isomers. The primary conclusion from this work is that with β-CD in the mobile phase very significant improvements in the separation of hydroxyl aromatics can be achieved.

ACKNOWLEDGEMENTS

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CHROM. 22 470

High-performance liquid chromatographic determination of malonaldehyde using *p*-nitrophenylhydrazine as a derivatizing reagent

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ABSTRACT

A high-performance liquid chromatographic procedure for the specific determination of free malonaldehyde (MA) is described. MA solution was mixed with 1 *M* acetate buffer (pH 3.76) and was reacted with *p*-nitrophenylhydrazine (NPH) hydrochloride solution in ethanol. An aliquot of the reaction mixture was injected into a C_{18}-5 column with a mobile phase consisting of acetonitrile–isopropanol–0.01 *M* sodium dihydrogenphosphate (30:10:60, v/v/v) and detection at 315 nm. MA reacted readily with NPH in a weakly acidic medium at room temperature, giving 1-(*p*-nitrophenyl)pyrazole. The method is specific for free MA. The response was linear in the range 36–720 ng/ml of MA and the detection limit was 6 ng/ml with a 20-μl injection.

INTRODUCTION

Lipid peroxidation has been considered to be a basic reaction involved in membrane damage. Malonaldehyde (MA) is a by-product of lipid peroxidation and its measurement has been suggested as being useful as an indicator of cellular ageing and free radical damage.

There are several high-performance liquid chromatographic (HPLC) methods for monitoring MA derivatized with 2-thiobarbituric acid (TBA)[1,2], dansylhydrazine (DNSH)[3] or 2,4-dinitrophenylhydrazine (DNPH)[4,5] or by the Hantzsch reaction[6]. Reactions of MA with TBA or DNSH require hot and acidic conditions, and the HPLC of the reaction products requires the employment of visible detection. A major drawback of these methods from a biochemical point of view is the artifactual generation of MA from the biological materials that can be induced under the strongly acidic conditions and elevated temperatures required for the formation of the derivatives. In a previous paper[5], we reported an HPLC method using DNPH for the

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determination of MA; where the reaction proceeded readily at room temperature and HPLC with UV detection could be used.

Recently, we found *p*-nitrophenylhydrazine (NPH) to have different advantages to DNPH as a derivatizing reagent for MA: (1) DNPH reacts with MA in a strongly acidic medium, whereas NPH reacts in a weakly acidic medium at room temperature, i.e., under milder conditions, which means that the procedure is specific for free MA; and (2) MA-NPH standard has higher molar absorptivity than that of MA-DNPH at their respective λ_{max}, 315 and 305 nm, and therefore the use of NPH is more suitable for the determination of trace amounts of MA. In this paper, we propose a specific and sensitive HPLC method for the determination of free MA.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile and isopropanol were purchased from Wako (Osaka, Japan), and 1,1,3,3-tetraethoxypropane (TEP), NPH hydrochloride (NPH · HCl) and 2-nitroresorcinol from Tokyo Kasei (Tokyo, Japan).

Preparation of MA standard solution

TEP (1 mmol) was dissolved in 100 ml of 0.01 *M* hydrochloric acid and the mixture was heated at 50°C for 1 h. This MA stock solution was kept at 4°C in the dark and the appropriate standard solution was made by dilution with water.

Preparation of MA-NPH standard

A standard of the derivative (MA-NPH) obtained by reaction of MA with NPH was prepared by heating at 50°C for 10 min a mixture of 20 ml of MA in 0.01 *M* hydrochloric acid (720 μg/ml) and 15 ml of NPH · HCl in 0.01 *M* hydrochloric acid (2 mg/ml). The mixture was extracted with 60 ml of ethyl acetate, the extract was washed with 10 ml of 0.01 *M* hydrochloric acid and three 10-ml volumes of water, dried over anhydrous sodium sulphate and evaporated to dryness. The residue was purified by sublimation under reduced pressure: yellow powder, m.p. 124–126°C; analysis, calculated for $C_9H_7O_2N_3$, C 57.14, H 3.73, N 22.21, found C 57.28, H 3.80, N 22.17%; λ_{max}, 315 nm; ε, 13400 in the mobile phase.

HPLC

A Model 5A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a UV spectrophotometric detector set at 315 nm and a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.) were used for quantification of MA. The HPLC separations were performed on a Develosil C_{18}-5 column (250 × 4.6 mm I.D.) (Nomura Chemical, Aichi, Japan) with a mobile phase consisting of acetonitrile–isopropanol–0.01 *M* sodium dihydrogenphosphate (30:10:60, v/v/v) at a flow-rate of 1.0 ml/min at room temperature.

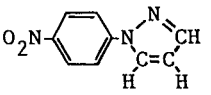
Procedure

A 1-ml volume of MA standard solution was mixed with 0.6 ml of 1 *M* acetate buffer solution (pH 3.76) and was reacted with 0.2 ml of NPH · HCl solution (0.5

mg/ml in ethanol) containing an appropriate amount of 2-nitroresorcinol as an internal standard (I.S.) at room temperature for 1 h. An aliquot of the reaction mixture was injected into the HPLC column. The concentration was calculated from a calibration graph obtained using mixtures of known amounts of MA-NPH standard and I.S.

RESULTS AND DISCUSSION

A typical separation of MA-NPH is illustrated in Fig. 1 using 2-nitroresorcinol as an I.S. Optimum HPLC conditions were established on the basis of a series of preliminary investigations. MA reacted readily with NPH in a weakly acidic medium giving 1-(*p*-nitrophenyl)pyrazole (MA-NPH), and the structure (I) was confirmed by mass spectrometry having M^+ (base peak) at *m/z* 189, and elemental analysis of the standard.



I

MA standard solution was prepared by acidic hydrolysis of 1,1,3,3-tetraethoxypropane (TEP). The amount of MA in a freshly prepared solution was measured by using the reaction with NPH in comparison with the MA-NPH standard

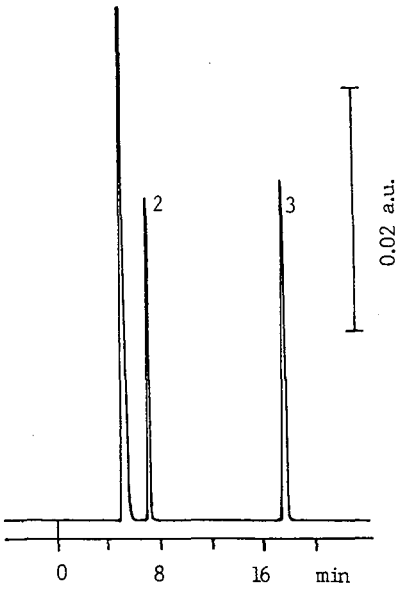


Fig. 1. Elution profile of MA (7.2 μg/ml) with a 10-μl injection after treatment with NPH reagent containing 16 μg of I.S. Peaks: 1 = NPH; 2 = I.S. (2-nitroresorcinol); 3 = MA-NPH.

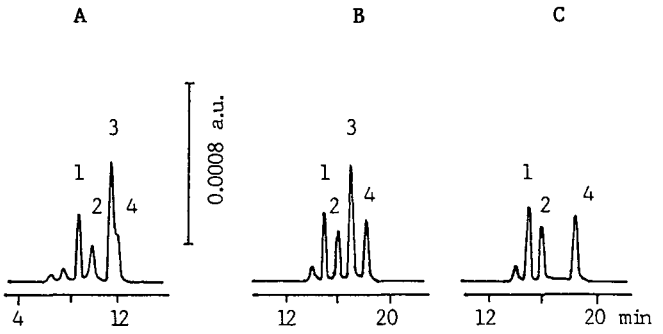


Fig. 2. Elution profile of MA (A and B, 100 ng/ml; C, blank) with a 20-μl injection after treatment with NPH reagent. Peaks: 1, 2, 4 = interfering peaks produced from NPH reagent; 3 = MA-NPH. Mobile phase: (A) acetonitrile–0.01 *M* hydrochloric acid (40:60, v/v), 1.5 ml/min; (B and C) acetonitrile–isopropanol–0.01 *M* sodium dihydrogenphosphate (30:10:60, v/v/v), 1.0 ml/min.

and the value corresponded to about 98% of that calculated from TEP taken assuming 100% conversion to MA. MA was relatively stable, but could not be stored for a long time in the pure state. It was found that the content of MA in the stock solution decreased to 85% after 2 months in a refrigerator and to 72% in the laboratory at room temperature. Therefore, it is advisable to prepare MA stock solution freshly once a week or to check the content of MA by comparison with MA-NPH standard.

MA-NPH standard was very easy to prepare and was used to construct calibration graphs. The yellow powder in the mobile phase showed λ_{max} at 315 nm, which was favourable for preventing interferences from various coexisting substances present in biological samples as well as MA-DNPH (λ_{max} = 305 nm), and the molar absorptivity (ε = 13400) at 315 nm was higher than that of MA-DNPH at 305 nm (ε = 10000), which was helpful for the trace determination of MA.

An NPH · HCl solution in ethanol was unstable and when a mixture of acetonitrile and 0.01 *M* hydrochloric acid (40:60, v/v) was used as the mobile phase some small peaks unfortunately appeared at a position close to the retention time of MA-NPH, as shown in Fig. 2A. In order to solve this problem in trace analysis, the effect of the composition of the mobile phase on the separation of MA-NPH from the interfering peaks was examined. The use of sodium dihydrogenphosphate which was better than hydrochloric acid for maintenance of the column, did not affect the separation, but addition of 10% isopropanol was found to improve the separation considerably (Fig. 2B). Fig. 2C shows the blank obtained from a solution without MA.

The effect of the pH of the reaction mixture on the reaction yield of MA and NPH was examined by using the ratio of the peak height to that of the I.S. and the results are shown in Fig. 3. MA is a weak acid (pK_a = 4.46)[7], which means that it exist dominantly as its conjugate base at >4.46:

$$\underset{\text{enol form}}{\text{OHC–CH=CHOH}} \overset{pK_a=4.46}{\rightleftharpoons} \underset{\text{conjugate base}}{\text{OHC–CH=CHO}^-}$$

The conjugate base is considered to be much less reactive than is MA toward NPH (nucleophile). The effect of pH at after 30 min of reaction (solid symbols)

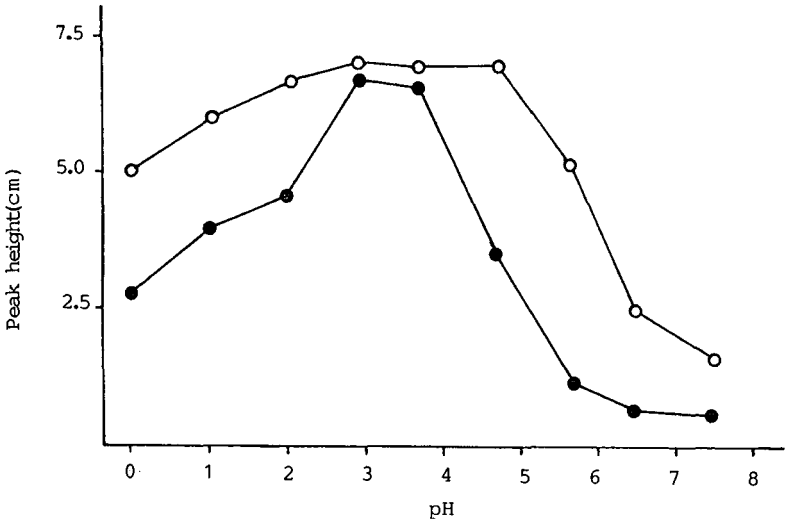


Fig. 3. Effect of pH on reaction yield of MA and NPH. A 1-ml volume of MA (7.2 μg/ml) was treated with 0.2 ml of NPH·HCl solution (0.5 mg/ml in ethanol) at various pH. Complex formation was determined by HPLC. (●) For 30 min at 25°C; (○) for 120 min at 25°C.

supports the above mechanism. However, the acidity of the reaction mixture hardly affected the reaction yield of MA and NPH at 120 min (open symbols) in the pH range 2.0–4.76. It is considered that the conjugate base which is present in equilibrium is converted to MA as the reaction of NPH and MA proceeds. MA reacted with DNPH in strongly acidic medium (pH <1)[5], whereas reaction with NPH decreased under these conditions. Reaction of MA with NPH involves nucleophilic attack by the basic nitrogen atom on the carbonyl carbon. Protonation of the carbonyl oxygen makes the carbonyl carbon more susceptible to nucleophilic attack. However, the nitrogen atom can also undergo protonation, removing unshared electrons, and is then no longer nucleophilic. The optimum conditions depend on the basicity of the reagent and on the reactivity of the carbonyl compound. The condensation reaction proceeded readily in a weakly acidic medium (pH 3.0–4.5) at room temperature to reach the maximum ratio in about 60 min. The effect of NPH concentration was also examined until there was no further increase in the amount of MA-NPH generated from the reaction. The final conditions adopted are those described under *Procedure*.

It has been postulated that MA exists in the free form, bound forms with various compounds and precursors in biological materials. A serious problem with methods for the determination of MA when applied in *in vivo* experiments is the possible formation of artificial MA during sample preparation. The amount of MA detected has been reported to be significantly influenced by the method of treatment of the sample. The procedures reported in previous papers required strongly acidic and/or hot conditions for the formation of derivatives. These methods were susceptible to artifactual generation of MA during the procedure. If the sample was treated under acidic and hot conditions, it is uncertain what proportion of the MA determined was originally present in the sample in the free state and what proportion may have been

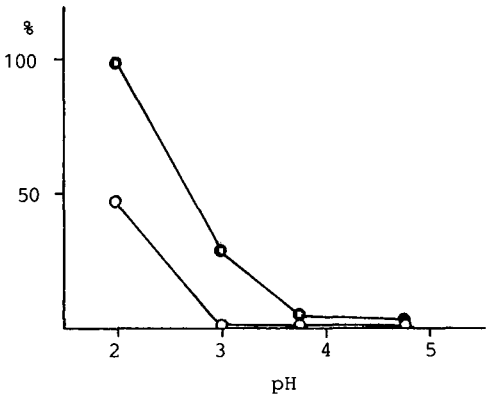


Fig. 4. Effect of pH on hydrolysis of TEP (0.1 μmol/ml): (○) at 25°C for 120 min; (●) at 50°C for 120 min. Formation of MA-NPH was determined by HPLC.

bound or may have been generated from precursors. Therefore, mild derivatization conditions are required for the reliable determination of free MA.

The reaction of MA and DNPH proceeded at room temperature, but required strongly acidic conditions (pH <1), as described previously[5], whereas the reaction with NPH proceeded under weakly acidic conditions (pH 2–5) at room temperature. In order to confirm that the present procedure is specific to free MA, 0.1 μmol/ml of TEP, a precursor of MA, was subjected to the reaction according to above procedure at pH 2, 3, 3.76 and 4.76 and 25 and 50°C, and the amounts of MA determined are shown in Fig. 4. At room temperature, about 48% of MA was liberated from TEP at pH 2, but at pH $\geqslant$ 3.76 little MA was hydrolysed from TEP even at 50°C. The results indicate that the present procedure is specific to free MA. Although the reaction of MA and NPH proceeded best at *ca.* pH 3, pH 3.76 was chosen as it is a milder condition and is also easier to adjust than pH 3.

The use of DNPH was not specific to free MA, because the conditions used were acidic enough (pH $\approx$0.5–1.0) to release MA from its precursors. Accordingly, by using the present method, the determination of free MA and that generated from its precursors is achieved from the difference between the MA levels prior to and after acid hydrolysis.

As described above, the concentration of MA in the standard solution has usually been calculated assuming 100% conversion of TEP to MA, but it is not easy to ensure an exact content of MA. Therefore, it is recommended to apply MA-NPH standard solution to construct a calibration graph for the reliable determination of MA and to standardize MA solutions.

With the reaction system used it is necessary to know the range over which MA can be determined and whether the formation of MA-NPH is linear over this range. A non-linear response indicates the possibility of a side-reaction. The response was linear in the range 36–720 ng/ml of MA in water and the correlation coefficient for the line was 0.998. The reproducibility of the method was determined by independently determining the amount of MA in six identical samples (720 ng/ml). The mean relative standard deviation was 1.4%. The detection limit at a signal-to-noise ratio of 2 was

6 ng/ml with a 20-μl injection, which was sufficient for the determination of urinary MA.

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CHROM. 22 498

Combination of adsorption and size-exclusion chromatography for the determination of fatty acid monomers, dimers and polymers

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ABSTRACT

A procedure based on adsorption and size-exclusion chromatography is proposed for evaluating fatty acid monomers, dimers and polymers in fats subjected to thermal oxidation. Starting from fatty acid methyl esters, two fractions of different polarity are obtained by silica column chromatography and submitted to a second separation by high-performance size-exclusion chromatography. The procedure allows the determination of unaltered fatty acids in addition to four groups of degradation compounds: non-polar fatty acid dimers, oxidized fatty acid monomers, polar fatty acid dimers and fatty acid polymers.

INTRODUCTION

The evaluation of minor components formed in heated or frying fats has always been difficult owing to the problems involved in their isolation and identification. However, advances in instrumental techniques have contributed to a better knowledge of the alterations that occur[1–3]. Several procedures for an objective evaluation of the alteration level in heated fats have been applied[4–8], including the determination of polar compounds proposed by IUPAC as a standard method for frying fats[4,9]. All these methods are based on the determination of the degradation compounds formed but give no idea about the structure or significance of such alteration compounds.

It is clear that a good definition of thermally oxidized fats must be based on the determination of alteration products, owing to their different chemical and nutritional significances. Some studies have contributed to this aim, but they are tedious and do not give quantitative results[10,11].

Previously, the possibilities of applying exclusion chromatography to the separation of alteration compounds produced during the heating of fats have been explored, and the direct analysis of fats[12–14] or their methyl ester derivatives[14–16] has been studied. The analysis is simple, as it is necessary only to dilute the fat or methyl esters in the appropriate solvent before the chromatographic determination, but the

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resolution and detection of minor compounds are very poor owing to the presence of unaltered triglycerides or unaltered methyl esters as major components.

Application of the technique to concentrated fractions from fats has been reported[12,17,18] and the possibilities of the elucidation of the degradation mechanisms have been considered using a method based on the combination of column and high-performance size-exclusion chromatography[19]. The latter method allows the determination of the global alteration level of a fat sample and the contributions of the main groups of compounds coming from hydrolytic, oxidative and thermal degradations to the total alteration; it is also possible to apply the method to a better definition of non-heated fats.

In this paper, a similar combination of adsorption and high-performance size-exclusion chromatography (HPSEC) is proposed for the determination of fatty acid methyl ester derivatives, which allows the elucidation of oxidation and thermal alteration mechanisms. The procedure permits the differentiation and quantification of five groups of compounds: unaltered fatty acids, non-polar fatty acid dimers, oxidized fatty acid monomers, polar fatty acid dimers and fatty acid polymers.

EXPERIMENTAL

Standards

Methyl palmitate, stearate and oleate (Sigma, St. Louis, MO, U.S.A.) and two mixtures of pure fatty acid methyl esters obtained from edible fats and oils of different unsaturation levels were used as standards for the determination of unaltered fatty acids by HPSEC.

Non-polar dimeric methyl esters obtained from linoleic acid heated at 220°C under nitrogen, isolated by column chromatography and subsequently purified by thin-layer chromatography (TLC)[20] were used as standards for the determination of non-polar dimers by HPSEC.

Oil samples

The following oil samples were analysed: refined olive and sunflower oils, both without heating and after heating at 190 ± 2°C for 100 h; and pure olive oil, unheated and heated at 180 ± 2°C for 150 h, and a 1:1 mixture of the two.

Procedure

Analysis of the oils involved the following stages:

(1) Transesterification of the oil samples with sodium methoxide and hydrochloric acid–methanol and subsequent quantitative recovery of methyl esters[6].

(2) Separation of methyl esters by silica column chromatography, following the method proposed by IUPAC[9], with two modifications: (a) the use of hexane–diethyl ether (88:12) to elute the non-polar fraction, which permitted the recovery of both the unaltered fatty acids and non-polar dimers, and (b) final elution of the column with methanol to improve the recovery of the sample. Both fractions should be checked by TLC to confirm the efficiency of the separation. TLC was carried out on plates coated with silica gel 60 G (Merck, Darmstadt, F.R.G.). The plates were developed using hexane–diethyl ether–acetic acid (80:20:1, v/v/v) and the spots were revealed by exposure to iodine vapour.

(3) Separation of non-polar and polar fractions by HPSEC. The samples were analysed in a Konik (Barcelona, Spain) Model 500 A chromatograph with a 10-μl sample loop, a Hewlett-Packard 1037 A refractive index detector and two 100- and 500-Å Ultrastyragel columns (Water Assoc., Milford, MA, U.S.A.), connected in series and operated at 35°C. The columns were 25 cm × 0.77 cm I.D., packed with porous, highly cross-linked styrene–divinylbenzene copolymer (10 μm). HPLC-grade tetrahydrofuran served as the mobile phase with a flow-rate of 0.5 ml/min and the sample concentration was between 15 and 20 mg/ml in tetrahydrofuran.

RESULTS AND DISCUSSION

Fig. 1 shows the analytical scheme applied to a thermally oxidized fat, where the efficiency of the separation by TLC is also shown. The non-polar fraction consists of two groups of compounds: unaltered fatty acids, which can also be defined as non-polar monomers, and non-polar dimers. With the polar fraction, three peaks can be observed corresponding to oxidized monomers, oxidized or polar dimers and polymers.

The main difference from previous studies consists in the differentiation of five groups of compounds instead of three, owing to the first separation by column chromatography. It permits first the concentration of minor altered fatty acids and second the separation of monomers and dimers into two groups of different significance. Thus, monomers are separated into non-polar compounds and the monomers originating via oxidation, in the first and second fractions, respectively. For dimers, the non-polar compounds, eluted in the first fraction, are representative of thermal alteration as there is no oxygen involved in their formation[20]. On the another hand, oxidative dimers can be independently determined in the polar fraction.

The calibration graphs for the HPSEC determination of non-polar monomers and non-polar dimers are shown in Fig. 2. For non-polar monomers, three samples with different concentrations of the five standards indicated above were prepared. For non-polar dimers, 24 samples of different concentrations of standards were used, as representative of the non-polar dimers present in thermally oxidized oils.

The calibration graphs were plotted as peak area (y) against amount (x), according to the following equations: for non-polar monomers or unaltered fatty acids,

$$y = 0.104x - 0.252 \quad (1)$$

and for non-polar dimers,

$$y = 0.115x - 0.121 \quad (2)$$

For eqn. 1, $r = 0.9889$, $n = 15$ and concentration range = 1–18 mg/ml, and for eqn. 2, $r = 0.9905$, $n = 24$ and concentration range = 1–12 mg/ml.

Unaltered fatty acids and non-polar dimers were determined using these calibration graphs. As these two groups of compounds differ both in polarity and molecular weight, it is not difficult to prepare representative standards. However, quantitative results for oxidized monomers, oxidized dimers and polymers included in

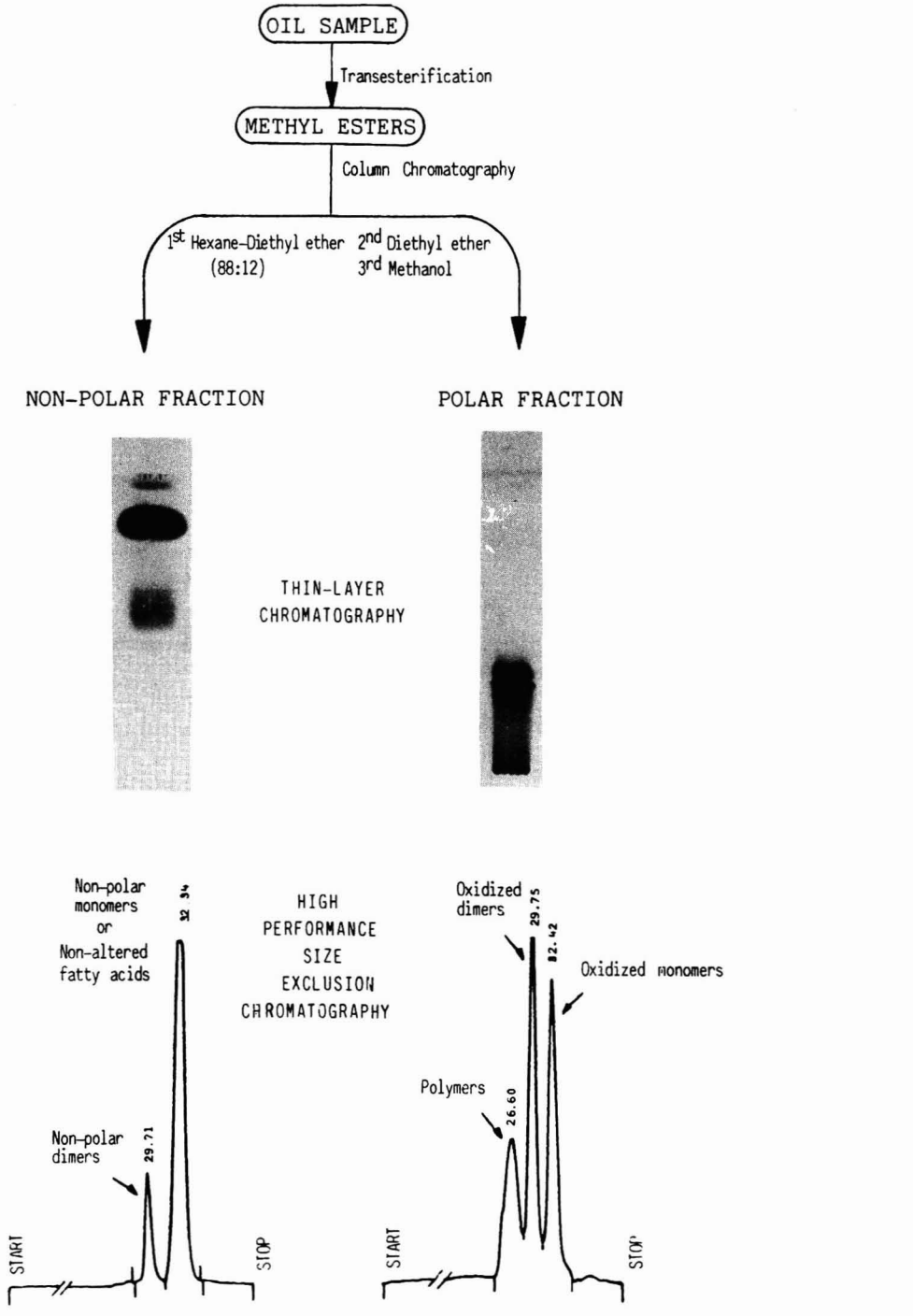

Fig. 1. Combination of column and size-exclusion chromatography for the determination of fatty acid methyl ester derivatives in heated fats.

the polar fraction were obtained by calculating their percentages relative to the total peak area. As it is known that compounds with very different chemical structures are involved in each peak[21,22], it is not possible to calculate precise response factors.

The determination of the five groups of compounds is shown in Table I. Each line lists the percentages of unaltered fatty acids or non-polar monomers, followed by those of the different groups of altered compounds. The last column shows the percentage of total altered fatty acids calculated by subtracting the percentage of unaltered fatty acid from 100. This is an interesting global measurement of acids undergoing thermal and oxidative alteration.

The results are means of four determinations in each instance. Limits of determination for the means ($\bar{x} \pm ts_{\bar{x}}$) can be easily deduced assuming a t distribution. The values of $ts_{\bar{x}}$ in Table I correspond to a 95% confidence level and three degrees of freedom ($t = 3.182$). As can be observed, reproducible results are obtained, in spite of the high value of t.

Polar compounds were not evaluated in unheated samples as their low proportion in comparison with the high content of unsaponifiable compounds eluting in the same fraction leads to difficulties in their determination.

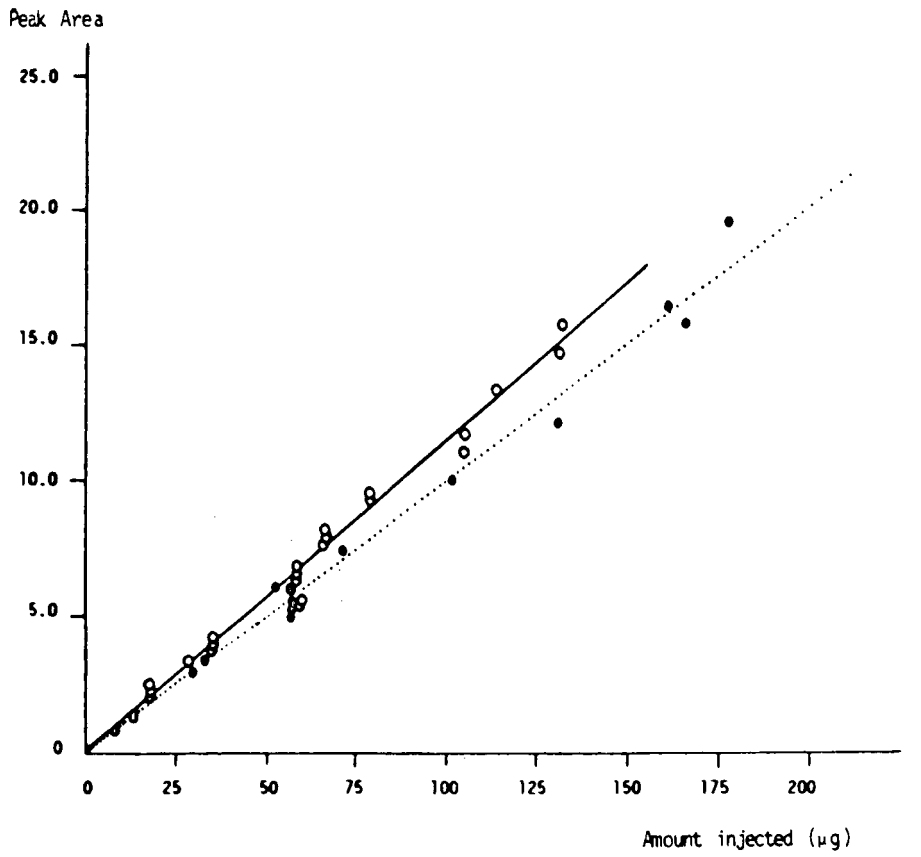

Fig. 2. Calibration graphs for (●) unaltered fatty acids and (○) non-polar dimers.

TABLE I

DETERMINATION OF NON-POLAR AND POLAR FATTY ACIDS IN THE INITIAL OILS AND AFTER HEATING (%, w/w ON FAT)

Sample	*Treatment*	*Non-polar fraction*				*Polar fraction*						*Total altered fatty acids*[c]
		Unaltered fatty acids		*Non-polar dimers*		*Oxidized monomers*		*Oxidized dimers*		*Polymers*		
		$\bar{x}$[a]	$ts_{\bar{x}}$[b]	$\bar{x}$	$ts_{\bar{x}}$	$\bar{x}$	$ts_{\bar{x}}$	$\bar{x}$	$ts_{\bar{x}}$	$\bar{x}$	$ts_{\bar{x}}$	
Refined olive oil	Unheated	98.7	0.5	–	–	–	–	–	–	–	–	2.3
	Heated	62.8	0.4	3.8	0.2	12.8	1.2	10.3	0.5	9.7	0.3	37.2
Refined sunflower oil	Unheated	97.5	0.3	–	–	–	–	–	–	–	–	2.5
	Heated	55.2	0.4	9.3	0.7	4.6	0.7	15.5	0.4	14.4	0.6	44.8
Pure olive oil	Unheated	98.3	0.5	–	–	–	–	–	–	–	–	1.7
	Heated	65.3	0.9	3.9	0.19	8.7	0.3	8.2	0.6	13.8	1.9	34.7
	Unheated–heated (1:1)	81.5	1.2	1.9	0.2	4.6	0.5	4.3	0.4	7.4	0.5	18.5

[a] Means of four determinations.
[b] $\bar{x} \pm ts_{\bar{x}}$: limits for the means (see text).
[c] Total altered fatty acids = 100 − unaltered fatty acids.

The results demonstrate that the proposed procedure permits comparisons to be made between samples with different unsaturation levels or subjected to different thermo-oxidative conditions. As can be observed, with similar heating conditions (100 h and 180°C) not only is the level of altered compounds higher in sunflower oil owing to its higher unsaturation, but also the distribution of such compounds is significantly different. Oxidized monomers reach higher percentages in olive oil whereas sunflower oil shows a greater concentration in of higher molecular weight compounds.

On the other hand, with similar unsaturation levels the greater percentage of polymers in the olive oil heated 150 h is notable, in spite of the similar altered fatty acid percentage. Hence the results clearly indicate that, indicating the alteration level of fats, the distribution of altered fatty acids provides useful information about the influence of oxygen and temperature on samples of unknown origin.

The main advantages of the proposed method are the following: a global measurement of thermo-oxidative alteration can be deduced from the contents of unaltered fatty acids; a substantial increase in the possibilities of the determination of polar compounds is obtained as oxidized and polymeric compounds can be determined without interference from less polar components, which are present in a majority; five groups of compounds (unaltered fatty acids, oxidized fatty acid monomers, non-polar fatty acid dimers, polar fatty acid dimers and fatty acid polymers) can be determined starting from the same sample; and it is possible to separate fatty acid dimers into two groups, non-polar and polar, and fatty acid monomers into unaltered and oxidized monomers. Non-polar dimers are specifically related to thermal degradation as there is no oxygen involved in their formation whereas oxidized monomers originate from the action of oxygen and their concentration in a sample is directly connected with oxidative alteration.

Finally, the proposed procedure can be combined with the similar evaluation proposed previously for glyceridic compounds[19] in order to obtain more information on fat alteration. This better chemical definition of thermally oxidized fats can be applied to nutritional studies involving edible frying fats, as it is well known that the oxidative and thermal degradations taking place in the unsaturated acyl groups of the triglyceride modify the physiological properties of the fat.

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Retention characteristics of CN, NH_2 and diol precoated high-performance thin-layer chromatographic plates in the adsorption and reversed-phase separation of some benzodiazepine derivatives

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ABSTRACT

The retentions of eighteen benzodiazepine (BZD) derivatives on CN, diol and NH_2 high-performance thin-layer chromatographic layers was determined using eight adsorption (carbon tetrachloride–ethyl acetate) and five reversed-phase (water–methanol) eluent systems. In most instances a linear correlation was found between the R_M value of BZD and the ethyl acetate or methanol concentration in the eluent, which allows the calculation of the optimum eluent composition. In aqueous eluents the lipophilicity of BZD determines the retention, that is, the plates behave as real reversed-phase stationary phases. The differences in the reversed-phase retentions of BZD were lower than in adsorption chromatography, advocating the application of adsorption chromatography for their separation. The low retention capacity and elongated spot shape make the NH_2 plate unsuitable for the reversed-phase separation of BZD without further modification of the composition of the mobile phase. As chlordiazepoxide and medazepam form distorted spots on CN and diol plates, for the best separation of these BZD, NH_2 plates in the adsorption separation mode are recommended.

INTRODUCTION

In recent years, high-performance thin-layer chromatography (HPTLC) has found growing acceptance and application among modern separation techniques[1]. Earlier results have already been reviewed[2]. Up to now mainly silica HPTLC plates have been applied for the separation of aflatoxins[3–5] and chlorthalidone and its impurities[6]. Amino phases have recently been applied to separate β-carotenes[7] and glucose and fructose[8].

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Benzodiazepine derivatives (BZD) have found growing application in modern therapeutic practice[9]. BZD are of considerable importance, having hypnotic, tranquillizing and anticonvulsant properties. As the range of BZD available has expanded rapidly over the last 10 years, many chromatographic methods have been developed for their separation and identification. The early separations were based on adsorption TLC[10,11] or pH-gradient TLC[12]. Earlier high-performance liquid chromatographic (HPLC) methods have been reviewed[13]. Both adsorption[14] and reversed-phase methods[15] have been used in the HPLC separation of BZD, and gas chromatography (GC) has als been frequently applied[16,17] sometimes with mass spectrometry[18]. The performances of the various chromatographic methods (TLC, GC and HPLC) have been compared[19].

The retention of a solute has been successfully predicted in binary eluent systems by various semi-empirical and theoretical approximations[20–25] using the various forms of eluent composition characteristics (preferably the concentration of the strong solvent in the eluent) as independent variables. The objectives of this work were to study and to compare the retention behaviours of some HPTLC layers in the adsorption and reversed-phase separation of some BZD.

EXPERIMENTAL

CN $F_{254}S$, NH_2 $F_{254}S$ and DIOL $F_{254}S$ HPTLC precoated plates (Merck, Darmstadt, F.R.G.) were applied both for adsorption and reversed-phase chromatography without any pretreatment.

The structures of BZD are given in Table I. The compounds were purchased from Hoffmann-La Roche (Basle, Switzerland) (compounds 1, 2, 11 and 14), Gedeon Richter (Budapest, Hungary) (compounds 3, 4, 6, 10, 13 and 15), Wyeth Labs. (Princetown, NJ, U.S.A.) (compound 5), VEB Arzneimittelwerk (Jena, G.D.R.) (compound 7), POLFA Pharmaceutical Works (Jelenia Góra, Poland) (compound 8), Upjohn Pharmaceutical Works (Kalamazoo, MI, U.S.A.) (compound 9), H. Mack Chemische Pharmazeutische Fabrik (Illertissen, F.R.G.) (compound 12), Gödecke (Frankfurt, F.R.G.) (compound 16), Hoechst (Frankfurt, F.R.G.) (compound 17) and EGIS Pharmaceutical Works (Budapest, Hungary) (compound 18). The BZD were separately dissolved in methanol at a concentration of 1 mg/ml and 500 nl of each solution were spotted onto the plates with a Camag (Muttenz, Switzerland) Micro Applicator. Both adsortion and reversed-phase developments were carried out in sandwich chambers of the same dimensions. Carbon tetrachloride–ethyl acetate mixtures served as eluents for adsorption chromatography in the concentration range 0–100% (v/v) ethyl acetate (eight eluent mixtures). Water–methanol mixtures were applied for the reversed-phase separation in the concentration range 0–80% (v/v) methanol (five eluent mixtures). After development the plates were dried at room temperature and the BZD spots were detected with a Shimadzu CS-930 dual-wavelength TLC scanner at 280 nm.

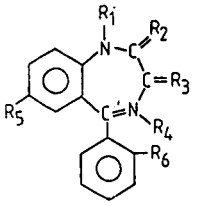
MATHEMATICAL METHODS

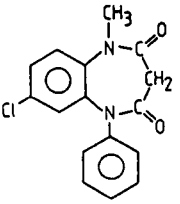
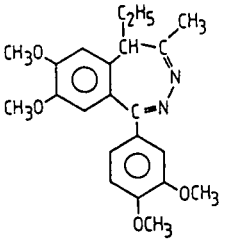
The R_M values were calculated separately for each BZD in each eluent:

$$R_M = \log (1/R_F - 1) \quad (1)$$

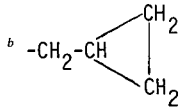
TABLE I

STRUCTURES OF BENZODIAZEPINE DERIVATIVES



Compound No.	*Common name*	R_1	R_2	R_3	R_4	R_5	R_6
1	7-Aminonitrozepam	H	=O	H	H	NH_2	H
2	Bromazepam	H	=O	H	H	Br	[a]
3	Uxepam	CH_3	=O	H	$CONH_2$	Cl	H
4	Oxazepam	H	=O	OH	H	H	H
5	Lorazepam	H	→O	H	H	Cl	Cl
6	Nitrazepam	H	=O	H	H	NO_2	H
7	Clonazepam	H	=O	H	H	NO_2	Cl
8	Chlordiazepoxide	H	$NHCH_3$	H	O	Cl	H
9	Alprazolam	$R_1C(CH_3)=NN=R_2$		H	H	Cl	H
10	Desmethyldiazepam	H	=O	H	H	Cl	H
11	Flunitrazepam	CH_3	=O	H	H	NO_2	F
12	Clorazepat	H	$(OH)_2$	COOH	H	Cl	H
13	Diazepam	CH_3	=O	H	H	Cl	H
14	Midazolam	$R_1C(CH_3)=NCH_2R_2$		H	H	Cl	F
15	Medazepam	CH_3	H	H	H	Cl	H
16	Prazepam	[b]	=O	H	H	Cl	H
17	Clobazam						
18	Tofisopam						

[a] 2-Pyridinyl instead of phenyl group.

[b] 

Linear correlations were calculated separately for each compound and for each plate type in both the adsorption and reversed-phase modes:

$$R_M = a + b \cdot C \tag{2}$$

where R_M = the actual R_M value of a compound determined at C%(v/v) ethyl acetate or methanol concentration; a = the R_M value of a compound extrapolated to zero ethyl acetate or methanol concentration; b = decrease in the R_M value caused by a 1% increase of ethyl acetate or methanol concentration; and C = ethyl acetate or methanol concentration (%,v/v).

On combination of eqns. 1 and 2, the R_F value, being the dependent variable, can be expressed as a function of the slope and intercept values of eqn. 2:

$$R_F = 1/(1 + 10^a \cdot 10^{bC}) \tag{3}$$

The optimum eluent composition for the separation of any pairs of BZD derivatives occurs when the R_F value expressed by eqn. 4 is a maximum:

$$R_F = 1/(1 + 10^{a_1} \cdot 10^{b_1 C}) - 1/(1 + 10^{a_2} \cdot 10^{b_2 C}) \tag{4}$$

where a_1, b_1, a_2 and b_2 are the intercept and the slope values of eqn. 2 for the BZD pair to be separated and C is the concentration (%, v/v) of ethyl acetate or methanol in the eluent. Eqn. 4 has a maximum when its first derivative equals zero.

To assess the real reversed-phase character of the separation in water–methanol mixtures, the intercept and slope values of BZD determined on CN and diol plates were linearly correlated with the corresponding values determined in traditional (silica support impregnated with 5% paraffin oil in *n*-hexane) reversed-phase chromatography[26].

The intercept (*a*) and slope (*b*) values of eqn. 2 characterize not only the retention behaviour of BZD but also the retention characteristics of the plates. To compare the BZD and the plate types, taking into consideration simultaneously their retention on each plate and in each eluent system, principal component analysis (PCA)[27] was applied. The parameters of eqn. 2 (intercept and slope values for CN, diol and NH_2 plates in the adsorption mode and intercept and slope values for CN and diol plates in the reversed-phase mode, total ten parameters) were taken as variables. The NH_2 plates in this instance were unsuitable for reversed-phase chromatography, so these values were excluded from the calculation. Individual BZD served for observations except compounds 14 and 15 (total 16 observations). The omission of compounds 14 and 15 from the calculations was motivated by the fact that eqn. 2 was not significant for compounds 14 and 15 on CN plates. To visualize the results, the two-dimensonal non-linear maps of PC loadings and variables were also calculated[28]. The non-linear mapping technique calculates the distances in the multi-dimensional space between the variables (parameters of eqn. 2) and observations (individual BZD), then it projects the relative distances in a plane with minimum error. In this way the distributions of parameters and BZD on the two-dimensional map are related to the PC loadings ad PC variables; they are derived from them but they are not identical with them. The axes F_1 and F_2 are on both maps coordinates without any concrete physical meaning. To

elucidate the impact of the physico-chemical parameters of BZD on their retention, the first three sets of PC variables containing the overwhelming majority of information was correlated with the lipophilicity, specific hydrophobic surface area[26] and pI value[29,30] of BZD. As the exact type of correlation between the dependent and independent variables had not previously been determined, we applied stepwise regression analysis[31] to overcome this difficulty. The PC variables were separately taken as dependent variables and the linear, logarithmic, quadratic and reciprocal forms of lipophilicity, specific hydrophobic surface area and pI values (total 12 variables) were taken as independent variables. The number of accepted variables was not limited; the acceptance limit for the individual independent variables was set to 95%.

RESULTS AND DISCUSSION

In most instances the BZD spots were symmetrical without any distortion. However, on NH_2 plates using the reversed-phase separation mode (eluent water), the BZD were fairly near to the front and had very elongated spots, that is, the NH_2 plates showed negligible retention capacity and therefore they are not suitable for the reversed-phase separation of BZD with the mobile phase used here. In adsorption chromatography compounds 8 and 15 produced elongated spots on diol and CN plates (Fig. 1) but not on NH_2 plates. This means that for the separation and determination of the BZD in mixtures containing these compounds the NH_2 plates are strongly recommended (for the separation possibilities of other derivatives, see the conclusions below).

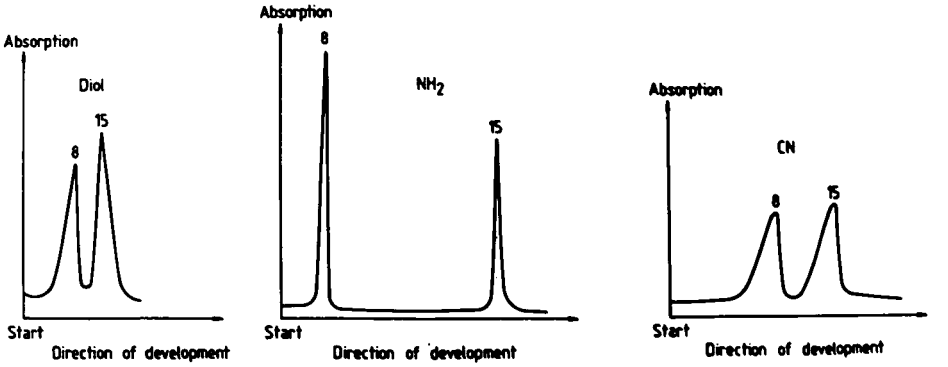


Fig. 1. Densitogram of the spots of compounds 8 and 15 (see Table I) on various layers. Eluent: CCl_4–ethyl acetate (1:1, v/v).

The dependence of the R_M values of some structurally different BZD on the eluent composition is shown in Figs. 2–4. The R_M values decreased in each instance with increasing concentration of ethyl acetate or methanol, that is, the BZD did not show any anomalous retention behaviour[32,33] which would make the application of eqn. 2 questionable. It also implies that the retention of BZD is governed in each instance by a single retention mechanism. The character of the dependence of the

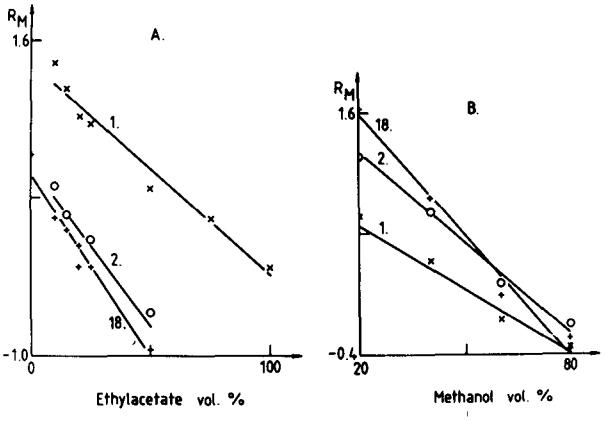


Fig. 2. Dependence of R_M value of some benzodiazepine derivatives on the ethyl acetate and methanol concentrations in CCl_4 and water, respectively. CN plates. Numbers refer to compounds in Table I.

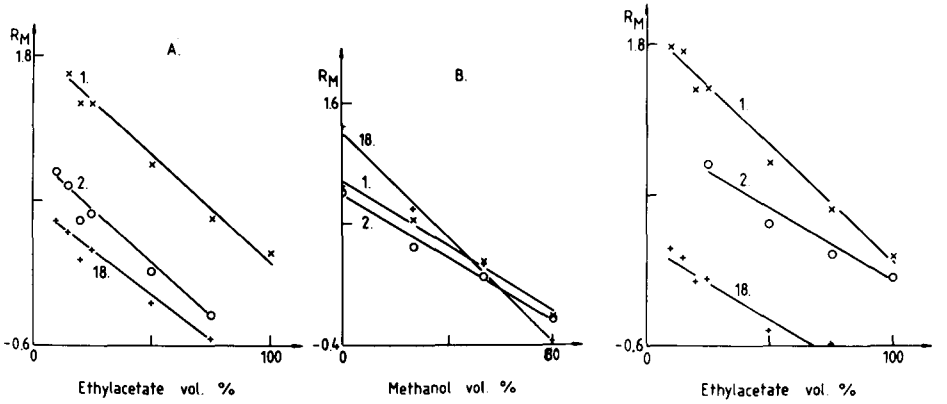


Fig. 3. Dependence of R_M values of some benzodiazepine derivatives on the ethyl acetate and methanol concentrations in CCl_4 and water respectively. Diol plates. Numbers refer to compounds in Table I.

Fig. 4. Dependence of R_M values of some benzodiazepine derivatives on the ethyl acetate concentration in CCl_4. NH_2 plates. Numbers refer to compounds in Table I.

retention on the eluent composition is different for the different plates, which means that the retention characteristics of the plates differ considerably. The parameters of the linear correlation between the R_M values of BZD and the eluent composition are given in Tables II–IV. In most instances the linear correlation fitted well to the experimental data, the significance level being over 95%. The high significance level of the correlation (low standard deviation of the slope and intercept values) makes it probable that the application, of eqn. 4 leads to a correct estimation of the optimum separation conditions. With CN plates the intercept and slope values show a higher diversity in adsorption than in the reversed-phase separation mode. This finding suggests that for the separation of BZD on CN plates adsorption chromatography is to be recommended. The same is valid, but to a lesser extent, to diol plates. We have to

TABLE II

PARAMETERS OF LINEAR CORRELATIONS BETWEEN THE R_M VALUES OF BENZODIAZEPINE DERIVATIVES AND THE ETHYL ACETATE (C_1) AND METHANOL (C_2) CONCENTRATIONS IN THE ELUENT WITH CN HPTLC PLATES

Compound No.	$R_M = a + b\,C_1$				$R_M = a + b\,C_2$			
	a	$-b$	s_b	r	a	$-b$	s_b	r
1	142.7	1.77	0.15	0.9834	115.5	1.87	0.17	0.9917
2	53.6	2.41	0.34	0.9715	170.5	2.36	0.16	0.9953
3	110.7	1.33	0.10	0.9856	168.5	2.50	0.20	0.9936
4	51.6	2.67	0.39	0.9696	167.5	2.46	0.20	0.9932
5	56.0	2.70	0.36	0.9747	208.5	2.93	0.21	0.9947
6	36.5	2.42	0.31	0.9769	194.5	2.57	0.36	0.9809
7	39.3	2.54	0.29	0.9807	210.0	2.84	0.22	0.9940
8	72.9	0.87	0.10	0.9666	202.0	2.83	0.40	0.9808
9	177.1	1.31	0.13	0.9771	195.5	2.65	0.20	0.9942
10	79.8	3.84	0.41	0.9776	195.5	2.74	0.21	0.9941
11	23.8	2.74	0.29	0.9785	215.0	2.84	0.28	0.9904
12	73.7	3.60	0.38	0.9783	196.0	2.76	0.22	0.9940
13	6.3	2.34	0.22	0.9829	214.5	2.89	0.22	0.9940
14		Not significant				Not significant		
15		Not significant			341.8	4.02	0.53	0.9913
16	13.3	5.22	0.65	0.9848	251.0	3.30	0.52	0.9878
17	66.1	2.90	0.32	0.9765	193.5	2.60	0.21	0.9933
18	51.6	3.07	0.34	0.9770	221.5	3.25	0.35	0.9888

TABLE III

PARAMETERS OF LINEAR CORRELATIONS BETWEEN THE R_M VALUES OF BENZODIAZEPINE DERIVATIVES AND THE ETHYL ACETATE (C_1) AND METHANOL (C_2) CONCENTRATIONS IN THE ELUENT WITH DIOL HPTLC PLATES

Compound No.	$R_M = a + b\,C_1$				$R_M = a + b\,C_2$			
	a	$-b$	s_b	r	a	$-b$	s_b	r
1	181.1	1.73	0.11	0.9917	94.5	1.77	0.15	0.9928
2	92.9	1.75	0.16	0.9831	82.2	1.69	0.14	0.9930
3	171.5	1.82	0.16	0.9815	68.5	1.52	0.12	0.9943
4	101.9	2.18	0.16	0.9893	78.4	1.63	0.13	0.9934
5	107.1	2.25	0.15	0.9909	88.8	1.83	0.14	0.9944
6	66.4	2.00	0.16	0.9878	71.4	1.48	0.13	0.9920
7	72.3	2.12	0.17	0.9870	87.9	1.75	0.16	0.9913
8	152.3	1.25	0.09	0.9877	119.8	1.78	0.25	0.9817
9	176.0	1.33	0.10	0.9857	104.1	2.09	0.12	0.9966
10	59.5	1.83	0.16	0.9846	103.4	2.03	0.16	0.9937
11	11.6	1.66	0.14	0.9868	75.7	1.64	0.12	0.9943
12	57.8	1.78	0.16	0.9847	102.2	2.01	0.15	0.9942
13	−8.8	1.20	0.12	0.9809	102.4	2.03	0.16	0.9937
14	170.8	1.07	0.12	0.9826	166.1	1.97	0.38	0.9651
15	61.0	0.41	0.03	0.9862	187.4	2.03	0.34	0.9734
16	−21.9	1.51	0.15	0.9865	123.3	2.33	0.24	0.9899
17	46.0	2.42	0.23	0.9869	69.8	1.61	0.09	0.9967
18	52.2	1.45	0.14	0.9824	138.4	2.90	0.17	0.9967

TABLE IV

PARAMETERS OF LINEAR CORRELATIONS BETWEEN THE R_M VALUE OF BENZODIAZEPINE DERIVATIVES AND THE ETHYL ACETATE (C_1) CONCENTRATION IN THE ELUENT WITH NH_2 HPTLC PLATES

Compound No.	$R_M = a + b\,C_1$			
	a	$-b$	s_b	r
1	191.0	1.88	0.11	0.9908
2	106.0	1.17	0.19	0.9735
3	199.3	1.85	0.11	0.9931
4	206.2	1.38	0.10	0.9894
5	227.0	1.58	0.25	0.9759
6	151.2	1.59	0.11	0.9883
7	144.8	1.54	0.09	0.9911
8	158.3	1.28	0.09	0.9874
9	150.6	1.59	0.17	0.9732
10	106.4	1.43	0.11	0.9861
11	37.3	2.20	0.19	0.9887
12	106.6	1.43	0.10	0.9879
13	5.5	1.84	0.17	0.9880
14	57.3	1.00	0.08	0.9843
15	Not significant			
16	−15.2	1.82	0.24	0.9747
17	44.8	1.38	0.09	0.9901
18	21.6	1.12	0.10	0.9819

stress that the conclusion outlined above are valid only for the compounds investigated and do not contain any information about the retention behaviour of the plates with other solutes. The significant correlations between the corresponding reversed-phase parameters (Table V) prove that both CN and diol plates behave as reversed phases in aqueous eluents, that is, the retention of solutes is governed mainly by their lipophilicity. Although the regression coefficients indicate significant linear correlations they are not high enough to allow the application of CN or diol plates for the determination of the lipophilicity of BZD.

TABLE V

PARAMETERS OF LINEAR CORRELATIONS BETWEEN THE INTERCEPT AND SLOPE VALUES OF BENZODIAZEPINES DETERMINED ON VARIOUS PLATES IN THE REVERSED-PHASE MODE

$y = a + bxn$				n	a	b	s_b	r
y		x						
Parameter	*Plate type*	*Parameter*	*Plate type*					
Intercept	Diol	Intercept	rp	18	1.89	31.95	7.09	0.7480
Slope	Diol	Slope	rp	18	0.64	0.16	0.05	0.6478
Intercept	CN	Intercept	rp	17	36.18	54.25	10.44	0.8017
Slope	CN	Slope	rp	17	0.72	0.26	0.07	0.6885

[a] rp = Traditional reversed-phase plate.

Principal component analysis proved that the ten variables can be substituted with three hypothetical ones that explain more than 80% of the total variance (Table VI). PC loadings show that each parameter of the CN plates and many parameters of the diol and NH_2 plates have a high loading in the first PC, that is, this PC can be tentatively identified with the retention characteristics of CN plates and with those retention characteristics of diol and NH_2 plates which are similar to those of CN plates. The second PC consists mainly of the high loadings of diol plates, so it can be considered as a background variable with diol-type retention characteristics.

TABLE VI

RESULTS OF PRINCIPAL COMPONENTS ANALYSIS

Principal component No.	*Eigenvalue*	*Variance explained (%)*
1	4.90	49.00
2	2.19	21.94
3	1.20	11.96
4	0.69	6.86

Principal component loadings

Variable No.	*Principal component No.*			
	1	*2*	*3*	*4*
1	−0.66	0.58	−0.04	0.23
2	0.71	−0.36	0.24	0.47
3	0.88	−0.09	−0.01	−0.38
4	0.89	0.06	0.08	−0.35
5	−0.84	0.49	0.07	−0.12
6	−0.35	−0.68	0.55	0.08
7	0.62	0.73	0.08	0.07
8	0.71	0.60	0.14	0.23
9	−0.83	0.08	0.24	−0.24
10	−0.05	−0.33	−0.86	0.11

The results of stepwise regression analysis proved that the first PC variable correlated quadratically with the specific hydrophobic surface area of BZD ($a = -5.75$; $b = 0.09$; $s_b = 0.02$; $r = 0.7812$), that is, this physico-chemical parameter has the greatest influence for the first background variable. With the second and third PC variables no significant correlation was found, that is, these variables are influenced to physico-chemical parameters that were not included in the calculations.

On the two-dimensional non-linear map of PC loadings (Fig. 5) the intercept (points 1, 5 and 9) and slope (points 2, 6 and 10) values of eqn. 2 calculated from the adsorption chromatographic data for each plate type form distinct groups. We again draw attention to the fact that the coordinates of the two-dimensional maps (F_1 and F_2) were calculated from the PC loadings and PC variables, but F_1 and F_2 are not identical (or simply related) to the PC loadings in Table VI and PC variables (not

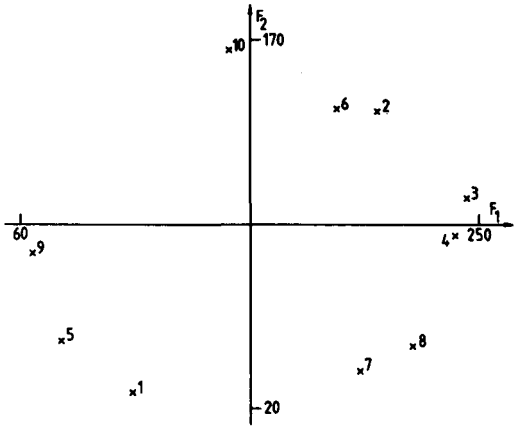


Fig. 5. Two-dimensional non-linear map of PC loadings. Number of iterations, 51; maximum error, 2.82 · 10^{-2}. Numbers refer to the parameters of eqn. 2. Intercept (1) and slope (2) values on CN plates, adsorption system; intercept (3) and slope (4) values on CN plates, reversed-phase system; intercept (5) and slope (6) values on diol plates, adsorption system; intercept (7) and slope (8) values on diol plates, reversed-phase system; intercept (9) and slope (10) values on NH_2 plates, adsorption system.

given). The four reversed-phase parameters (points 3, 4, 7 and 8) also form a cluster. This finding proves again that the plates show different retention capacities and selectivities in adsorption and reversed-phase chromatography. The BZD do not form separate clusters according to either the number or the type of substituents on the two-dimensional non-linear map of PC variables (Fig. 6). This result indicates that each substituent has a similar impact on the retention. The map also contains information about the separation capacity of the CN, diol and NH_2 plates for BZD. The greater the difference between two BZD the higher is the probability that they can be separated in one or more chromatographic systems. The very close positions of compounds 10 and 12 on the map indicate that they can hardly be separated with the chromatographic systems applied.

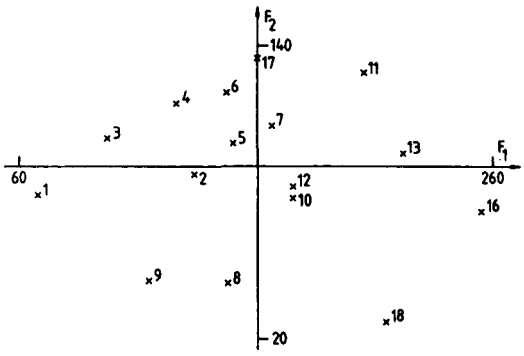


Fig. 6. Two-dimensional non-linear map of PC variables. Numbers refer to benzodiazepine derivatives in Table I. Number of iterations, 103; maximum error, 2.82 · 10^{-2}.

CONCLUSIONS

CN, diol and NH_2 HPTLC plates seem to be suitable for the separation of most benzodiazepine derivatives. Except for the NH_2 plates, the separation can be carried out both with adsorption (carbon tetrachloride–ethyl-acetate) and reversed-phase (methanol–water) eluents, but the separation efficiency was higher in adsorption than in reversed-phase systems in all instances.

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Capillary zone electrophoresis of pyridylamino derivatives of maltooligosaccharides

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ABSTRACT

Maltooligosaccharides derivatized with 2-aminopyridine were separated by capillary zone electrophoresis in the pH range 3.0–4.5 using 0.1 *M* phosphate solutions as the running electrolyte. The inclusion of small amounts of tetrabutylammonium bromide in the electrolyte solution facilitated the separation at pH 5.0 and yielded high separation efficiency. The separated zones of pyridylamino derivatives of maltooligosaccharides migrated across the fused-silica capillary and passed the detection point in the order of increasing size. The "overall mobility" was a linear function of the number of glucose residues in the homologous series.

INTRODUCTION

Capillary zone electrophoresis (CZE) is increasingly employed for the separation and analysis of a wide variety of compounds ranging in size from small ions and molecules[1–7] to high-molecular-weight substances such as proteins[8–10] and nucleic acid fragments[11,12]. However, the potential of the technique has not been yet exploited in many areas including carbohydrates. This may be due to the lack of charges and chromophores in the carbohydrate molecules.

Indeed, neutral carbohydrates are not directly amenable to electrophoresis. However, they may become charged as the result of complex formation with other ions. The complex formation equilibria, *e.g.* borate complexes, have been exploited in traditional paper electrophoresis[13,14]. Very recently, the sugar-borate complexes at pH 10 have been utilized in CZE for the separation of reducing monosaccharides[15] tagged with 2-aminopyridine.

On the other hand, pre-column derivatizations to produce a chromophore or fluorophore have been extensively used in high-performance liquid chromatography (HPLC) of carbohydrates to allow their sensitive detection[16,17] (for review see refs. 18 and 19). It is expected that many of these detection schemes will also be applied to carbohydrate detection in CZE.

In the present report, we extend the potential of CZE to the separation of pyridylamino (PA) derivatives of maltooligosaccharides. The derivatization of mono-

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and oligosaccharides with 2-aminopyridine is well documented[15,20] and was used for HPLC analysis of carbohydrates by UV[21] or fluorescence detection[17]. The tagging of maltooligosaccharides with 2-aminopyridine provides the homologues with a positive charge, which allowed their analysis by paper electrophoresis[20]. Indeed, the pK_a value of the derivatives has been found to be 6.7 (ref. 20), a value equal to that found for free 2-aminopyridine[22].

This paper presents the results of a study of the electrophoretic conditions for the separation of PA derivatives of maltooligosaccharides by CZE. High separation efficiencies were obtained in the pH range 3.0–4.5, which is ideal for the operation of fused-silica capillary columns as far as the stability of the column and the reproducibility of the separations are concerned. In addition, the inclusion of small amounts of tetrabutylammonium bromide in the running electrolyte was a useful adjunct to affect full separation of the homologous series at pH 5.0. The PA derivatives of the maltooligosaccharides eluted in the order of increasing size. The "overall mobility" was a linear function of the number of glucose residues in the homologous series. The slope of the lines, which we refer to as "overall mobility" decrement, is expected to facilitate the identification of oligosaccharides at large.

EXPERIMENTAL

Electropherograph

The instrument for capillary electrophoresis used in this study resembled that reported by others[3,4]. It was constructed from a Glassman High Voltage (Whitehouse Station, NJ, U.S.A.) Model EH30P3 high-voltage power supply and a Linear (Reno, NV, U.S.A.) Model 200 UV–VIS variable-wavelength detector equipped with a cell for on-column capillary detection. The detection wavelength was set at 240 nm. The electropherograms were recorded with a Kipp and Zonen Model BD 40 strip chart recorder. The high-voltage output and the ground were connected to platinum electrodes, which were placed in 2.0 ml electrolyte reservoirs. The ends of the fused-silica capillary were dipped in both reservoirs and that completed the circuit. The separation and sample introduction were carried out in the positive polarity mode, *i.e.* the anodic reservoir served as the high-voltage output (injection end) and the cathodic reservoir was at the ground potential (detection end).

Capillary columns

Fused-silica capillary columns of 50 μm I.D. and 365 μm O.D. having polyimide-clad were obtained from Polymicro Technology (Phoenix, AZ, U.S.A.). The total column length was 80 cm while the separation distance was 50 cm, *i.e.* from the injection end to the detection point. The polyimide-clad of the capillary was burned off at the detection point and the exposed quartz tubing was placed in the detector cell.

Reagents and materials

The following PA derivatives of maltooligosaccharides: PA-maltotetraose (PA-G_4), PA-maltopentaose (PA-G_5), PA-maltohexaose (PA-G_6), and PA-maltoheptaose (PA-G_7) were a gift by Dr. A. Mort from the Biochemistry Department at Oklahoma State University. The covalent attachment of 2-aminopyridine to the reducing end of the maltooligosaccharides was carried out using the procedure of

reductive amination[20]. Reagent-grade sodium phosphate monobasic and dibasic, phosphoric acid, hydrochloric acid, sodium hydroxide, phenol and tetrabutylammonium bromide were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Distilled water was used to prepare the running electrolyte as well as the solutions used in column cleaning and pretreatment. All solutions were filtered with 0.2-μm UniPrep syringeless filters obtained from Genex (Gaithersburg, MD, U.S.A.) to avoid column plugging.

Procedures

In all experiments (except where indicated) the voltage drop across the capillary during electrophoresis was fixed at 20 kV, while injection was made by electromigration at 18 kV for 15 s.

All experiments were carried out with uncoated fused-silica capillaries. The untreated silica capillary column was flushed with 1 *M* sodium hydroxide followed by water and the running electrolyte. The running electrolyte was renewed after 5–6 runs, and the capillary column was flushed with fresh buffer before each injection in order to ensure reproducible separations[23].

The electroosmotic flow-rate was measured by the ratio of the volume of the capillary (volume of a cylinder in 50 cm length and 50 μm I.D.) and the time required for phenol, an inert tracer[9], to migrate the 50 cm distance between the injection end and the detection point.

The "overall mobility" of the solute's zone is the sum of the electrophoretic mobility and the electroosmotic mobility; in this study both are directed toward the negative electrode. The "overall mobility," $\mu_{overall}$, is given by the equation[4], $\mu_{overall} = L^2/Vt$, where L, V and t are the total length of the capillary tube, the applied voltage, and the time for the zone to migrate the entire length of the tube, respectively. In CZE and using on column detection, the time t is given by the equation, $t = t_d L/l$, where l = distance from injection to the detector, and t_d = time for species to migrate the distance, which is readily obtained from the electropherogram. Substituting t in the above equation yields $\mu_{overall} = Ll/Vt_d$, which was used to measure the overall mobility.

RESULTS AND DISCUSSION

The electrophoretic behavior of each PA-maltooligosaccharide was examined using 0.1 *M* aqueous phosphate solutions at different pH values. The results are depicted in Fig. 1 in terms of migration time *versus* pH of the electrolyte solution. As expected, the migration time of the different homologues from the injection to the detection point decreased with increasing pH. This behavior is primarily due to the increase in electroosmotic flow[24], because higher electrolyte pH produces an increase in the ionization of silanol groups on the surface of fused-silica capillaries. Indeed, as can be seen in Figs. 1 and 2 phenol which is believed to migrate with the electroosmotic or bulk flow[9] clearly demonstrated this trend. In the pH range from 3.0 to 5.5 the positively charged PA-maltooligosaccharides migrated ahead of phenol and were separated according to their size. At pH 6.0 and 7.0, the derivatives practically coeluted at approximately the same migration time as phenol (see Fig. 1). Obviously in this pH range the PA-maltooligosaccharides become less positively charged. Under these

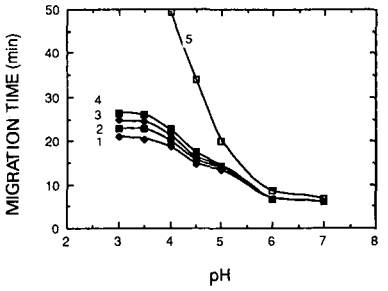


Fig. 1. Migration times of PA-maltooligosaccharides and phenol as a function of electrolyte pH. Capillary, fused-silica tube, 50 cm (to the detection point) × 50 μm I.D.; electrolytes, 0.1 *M* phosphate solutions at different pH values; voltage, 20 kV; current was *ca.* 60 μA; injection by electromigration for 15 s at 18 kV; temperature, 25°C. 1 = PA-G_4; 2 = PA-G_5; 3 = PA-G_6; 4 = PA-G_7; 5 = phenol.

circumstances neutral and quasi-neutral solutes are swept with the electroosmotic flow (bulk flow).

Fig. 3 illustrates the high separation efficiency obtained with a 50-cm (separation distance) untreated fused-silica capillary tube at 20 kV using 0.1 *M* phosphate, pH 4.0, as the running electrolyte. As can be seen in Fig. 3, base line resolution is obtained in less than 25 min.

Fig. 4 illustrates the "overall mobility" of PA-maltooligosaccharides at different pH values as a function of the number of glucose residues in the homologous series. The slope of the lines, which is the "overall mobility" decrement, δ, was relatively constant over the pH range 3.0 to 4.5 (see Table I). On the other hand, δ dropped sharply at pH 5.0, and reached almost zero at pH 6.0 and 7.0 whereby the PA-maltooligosaccharides moved virtually together with the bulk flow.

The effect of electrolyte concentration on the electrophoretic behavior of the homologues was studied at pH 4.0 using 0.025, 0.05, 0.075 and 0.1 *M* phosphate solutions. The results are depicted in Fig. 5 in terms of "overall mobility" *versus* the molar concentration of the running electrolyte. As expected, the "overall mobility" decreased with increasing phosphate concentration in the running electrolyte solution. This is due to the reduction in electroosmotic flow[25], as a consequence of an increase in

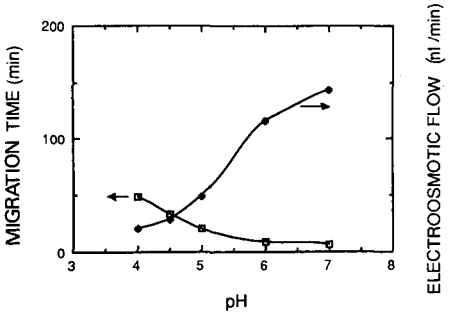
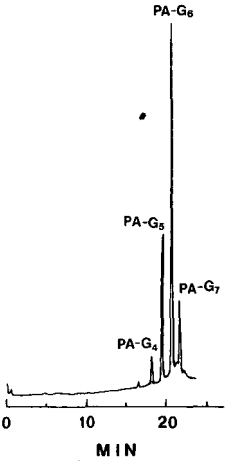


Fig. 2. Migration times for phenol and electroosmotic flow as a function of electrolyte pH. Other conditions as in Fig. 1.



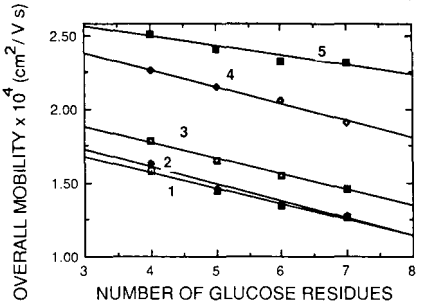


Fig. 3. Separation of PA-maltooligosaccharides. Electrolyte: 0.1 *M* phosphate, pH 4.0. UV detection at 240 nm. Other conditions as in Fig. 1.

Fig. 4. "Overall mobility" as a function of the number of glucose residues in the homologous series at different pH values. Other conditions as in Fig. 1. 1 = pH 3.0; 2 = pH 3.5; 3 = pH 4.0; 4 = pH 4.5; 5 = pH 5.0.

the viscosity of the electrolyte solution and a decrease in both the thickness of the double layer and the ζ potential of the capillary wall[26]. It has to be noted that a concentration of 0.075 to 0.1 *M* phosphate in the running electrolyte yielded sharper peaks and consequently higher resolution than a lower phosphate content. This may be due to the salt shielding effect of silanol groups on the capillary walls, which minimizes wall interaction with the positively charged PA-maltooligosaccharides.

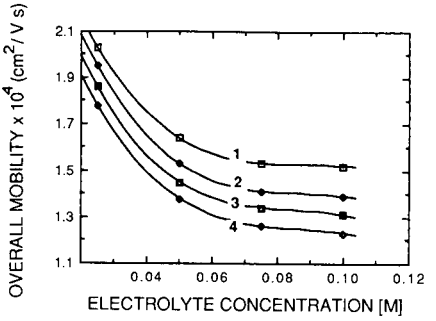
For each electrolyte concentration, the "overall mobility" data from the preceding experiments are plotted in Fig. 6 *versus* the number of glucose residues in the homologous series. Straight lines are obtained with a correlation coefficient equal or greater than 0.99 (see Table II). The "overall mobility" decrement, δ, for the homologues increased slightly with the electrolyte concentration by a factor of 1.13 when going from 0.025 to 0.1 *M* phosphate.

TABLE I

OVERALL MOBILITY DECREMENT, δ, AS ESTIMATED FROM THE SLOPE OF THE PLOTS OF OVERALL MOBILITY *VERSUS* THE NUMBER OF GLUCOSE RESIDUES IN THE HOMOLOGOUS SERIES

Electrolytes: 0.1 *M* phosphate solutions at different pH values.

pH	$\delta \cdot 10^6$ *cm²/Vs*	*Correlation coefficient*
3.0	−10.6	0.98
3.5	−11.5	0.97
4.0	−10.6	0.99
4.5	−11.4	0.99
5.0	− 6.5	0.92



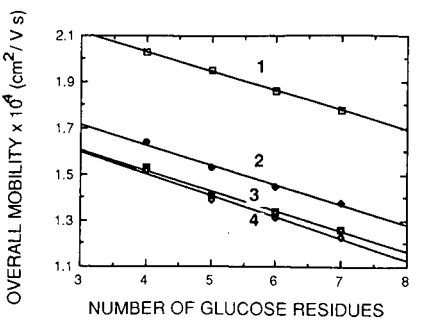


Fig. 5. "Overall mobility" as a function of electrolyte concentration. Electrolytes: phosphate solutions at different concentrations, pH 4.0. Currents: 15 μA, 25 μA, 40 μA and 60 μA at 0.025, 0.05, 0.075 and 0.1 *M*, respectively. Other conditions as in Fig. 1. 1 = PA-G_4; 2 = PA-G_5; 3 = PA-G_6; 4 = PA-G-.

Fig. 6. "Overall mobility" as a function of glucose residues in the homologous series at different phosphate concentrations in the electrolyte. 1 = 0.025 *M*; 2 = 0.05 *M*; 3 = 0.075 *M*; 4 = 0.1 *M*. Other conditions as in Fig. 5.

As indicated above the resolution between the homologues decreased with increasing pH. Fig. 7 shows the separation of PA-maltooligosaccharides at pH 5.0 with and without tetrabutylammonium bromide in the running electrolyte solution. The inclusion of 50 m*M* tetrabutylammonium bromide in the electrolyte solution yielded higher separation efficiency and concomitantly higher resolution. A further increase in resolution was achieved by using 15 kV instead of 20 kV. This may be due to a lesser heat overload of the system and decreased electroosmotic flow at the lower voltage.

To study the effect of tetrabutylammonium bromide on separation, 50 m*M* of this organic salt were added to 0.1 *M* phosphate solutions at different pH values. The results are summarized in Table III in terms of migration modulus, η, which is the ratio of the migration time of the solute in the presence to that in the absence of tetrabutylammonium bromide. At pH 5.0 and above, η is almost equal to unity, whereas at pH 4.5 and 3.0 η is greater than 1 and 2, respectively. In the pH range 5.0–7.0, the small changes in the migration of PA-maltooligosaccharides upon adding tetrabutylammonium bromide to the electrolyte solution may be viewed as the result of

TABLE II

OVERALL MOBILITY DECREMENT, δ, AS ESTIMATED FROM THE SLOPE OF THE PLOTS OF OVERALL MOBILITY *VERSUS* THE NUMBER OF GLUCOSE RESIDUES IN THE HOMOLOGOUS SERIES

Electrolytes: solutions at different phosphate concentrations, pH 4.0.

Phosphate concentration (M)	$\delta \cdot 10^6$ *cm²/Vs*	*Correlation coefficient*
0.025	−8.4	0.99
0.05	−8.6	0.99
0.075	−8.8	0.99
0.1	−9.5	0.99

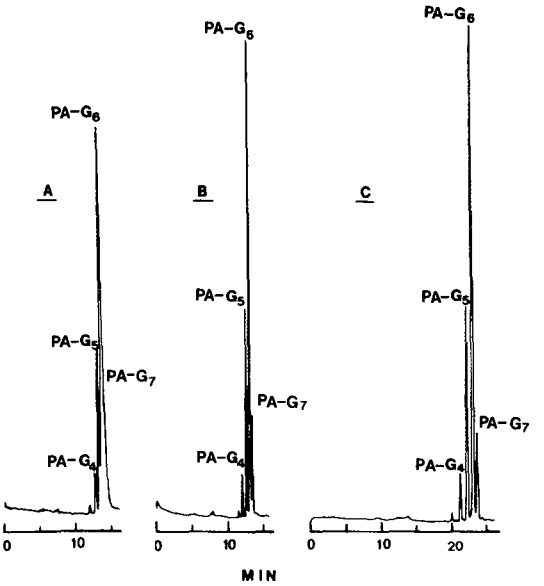


Fig. 7. Separation of PA-maltooligosaccharides. Electrolytes, 0.1 *M* phosphate, pH 5.0, without and with 50 m*M* tetrabutylammonium bromide in A and B, respectively. In both cases the voltage was 20 kV and the corresponding currents were 60 and 125 μA. In C as in B except voltage was 15 kV and current was 60 μA. Other conditions as in Fig. 1.

two opposing effects: increased overall mobility due to attenuation in wall interaction of the positively charged derivatives *versus* a reduction in the electroosmotic flow as a consequence of increasing the ionic strength of the electrolyte solution. At pH 3.0 and 4.5, where wall interaction is less pronounced, the enhancement in migration time upon adding the organic salt (see Table III) may be explained by the decrease in the electroosmotic flow resulting from an increase in the ionic strength of the running electrolyte.

TABLE III

MIGRATION MODULUS, η, OF PYRIDYLAMINO DERIVATIVES OF MALTOOLIGOSACCHARIDES AT DIFFERENT pH

Electrolytes: 0.1 *M* phosphate solutions with or without 50 m*M* tetrabutylammonium bromide.

PA-maltooligosaccharide	*Migration modulus, η*		
	pH 3.0	*pH 4.5*	*pH 5.0*
PA-G_4	1.8	1.5	0.96
PA-G_5	2.0	1.5	0.96
PA-G_6	2.3	1.5	0.96
PA-G_7	2.5	1.5	0.96

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CHROM. 22 491

Note

Gas chromatography of the atmosphere of Titan

II. Analysis of low-molecular-weight hydrocarbons and nitriles with a potassium chloride-deactivated alumina PLOT capillary column

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(First received December 1st, 1989; revised manuscript received April 3rd, 1990)

We are currently studying gas chromatographic (GC) columns which could be used for analysing the atmosphere of Titan, a satellite of Saturn, from a probe. In Part I[1], we described the chromatographic behaviour of a PoraPLOT Q column in the separation of C_1–C_8 hydrocarbons and C_1–C_4 nitriles[1]. We now present the results obtained for the same type of solutes with a potassium chloride-deactivated alumina (Al_2O_3–KCl) porous-layer open-tubular (PLOT) capillary column.

Such a column has already been systematically studied for the analysis of hydrocarbons[2–4]. It is very powerful for the separation of C_1–C_{10} hydrocarbons, including alkanes and ethylenic, acetylenic and benzenic compounds. As observed for other PLOT columns, the optimum velocity of the carrier gas is much higher than those obtained with liquid phase coated capillary columns, and for hydrogen it is about twice that for helium. In addition, it was found that this column shows a very high retention for polar compounds, such as alcohols[2].

However, no quantitative information was available on the behaviour of this column with low-molecular-weight nitriles. For that reason we have studied the separation of mixtures of hydrocarbons and nitriles and the height equivalent to a theoretical plate (HETP) of some of these compounds with the Al_2O_3–KCl PLOT column.

EXPERIMENTAL

The PLOT column (Chrompack, Middelburg, The Netherlands) was of 0.32 mm I.D. It was initially 50 m long and then it was reduced to 10 m. This fused-silica capillary PLOT column was coated with a 5-μm thick film of aluminium oxide (deactivated by potassium chloride for increased linear adsorption and more symmetrical peaks). The column was mounted in the split mode (splitting ratio 25:1)

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on the same gas chromatograph as described in Part I[1]. The injector and detector temperatures were 225°C. Hydrogen was used as the carrier gas.

The sampling techniques, reagents and method of determination of the Van Deemter curves were the same as in Part I[1].

RESULTS AND DISCUSSION

A 50-m column of this type was tested initially by injecting some nitriles independently. During these preliminary studies, hydrocyanic acid (HCN), cyanogen (NCCN), cyanoacetylene (HC_2CN), cyanopropyne (CH_3C_2CN) and acetonitrile (CH_3CN) were not eluted, even after several hours at high temperature (*ca.* 200°C). It appears therefore, that the adsorption of these compounds on the alumina surface is very important, probably as a consequence of strong interactions between nitriles, which are Lewis bases, and alumina, which is a Lewis acid. We therefore shortened the column to 10 m in order to optimize the analysis time and observe the elution of nitriles. With this shorter column, the elution of all the nitriles studied may be possible within reasonable times. However, some of them are effectively eluted (mainly HCN, C_2N_2 and the acetylenic nitriles) only if they reinjected in relatively large amounts. For instance, with C_2N_2, the minimum injected amount necessary to achieve elution of the solute is about 50 nmol (corresponding to 2 nmol injected onto the column, because of the splitting ratio). For HCN this amount is about 150 nmol (corresponding to 6 nmol injected onto the column). Such behaviour is probably due to irreversible adsorption

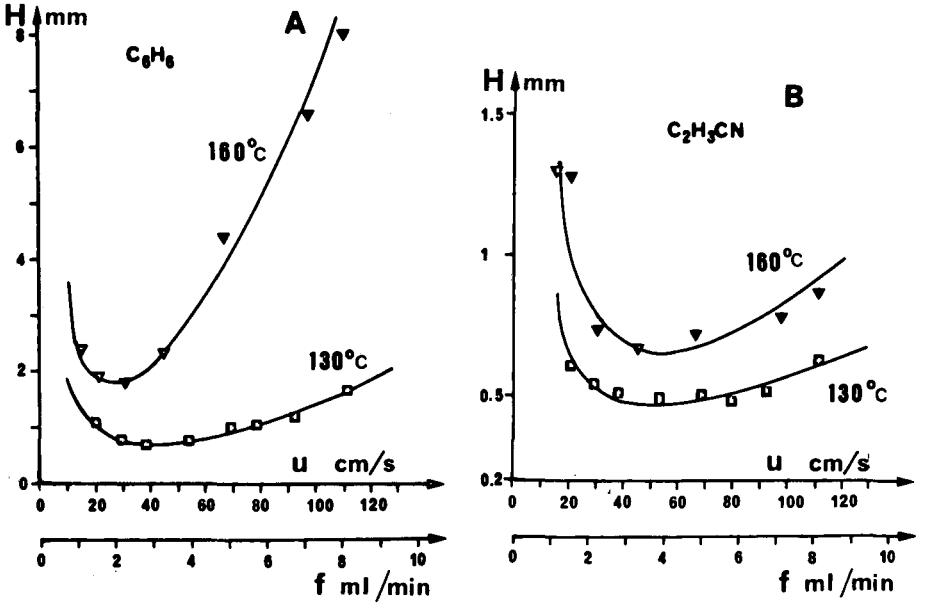

Fig. 1. Variation of the HETP (H) with the mean linear velocity (u) and outlet flow-rate (f) of the carrier gas (hydrogen) at 130 and 160°C on a 10 m × 0.32 mm I.D. fused-silica Al_2O_3–KCl PLOT column (5 μm thickness coating) for (A) benzene and (B) acrylonitrile. Total injected amount, benzene 0.6 nmol, acrylonitrile 0.9 nmol; split mode (25:1).

of part of the nitrile and not to chemical reaction with the coating material, as no degradation product was observed during the chromatographic analysis.

We determined the efficiency of the 10-m Al_2O_3–KCl PLOT capillary column long by measuring the HETP as a function of the linear gas velocity at different temperatures. To plot Van Deemter curves we injected a gas mixture of selected solutes (including in particular methane, benzene and acrylonitrile) at 130 and 160°C.

Fig. 1A shows the curves relating to benzene. The minimum values of H are 0.7 mm at 130°C and 1.8 mm at 160°C. In contrast to the results obtained with PoraPLOT Q[1], the HETP depends markedly on temperature. When the temperature is increased, a large increase in the slope of the linear portion of the HETP *vs.* u curve is observed at high velocities of the carrier gas. This shows that the C_k term of the Golay equation (HETP = $B/u + C_g u + C_k u$), which is related to resistance to mass transfer, increases when the temperature increases. This phenomenon may be due to an increase in diffusion into the micropores of the coating material of the column[5,6]. It has also been observed when chemi-adsorption processes are involved[7].

The curves for acrylonitrile are shown in Fig. 1B. The column efficiency for this polar compound is much better than that of the PoraPLOT Q column. The minimum values of H are 0.47 mm at 130°C and 0.65 mm at 160°C. With these two latter components, benzene and acetonitrile, we have observed that the column efficiency increases when the temperature decreases in the range of temperatures tested.

For the calibration of the column with C_6 hydrocarbons and C_1–C_4 nitriles, we used an outlet flow-rate of the carrier gas at 4.4 ml/min. The nitriles are generally eluted after the C_1–C_6 hydrocarbons. Therefore, the calibration of this column was carried out by injecting a mixture of methane, C_6 hydrocarbons (cyclohexane, 1-hexene and benzene) and C_1–C_4 nitriles at different temperatures.

Fig. 2A shows a chromatogram of such a mixture at 150°C. All of the injected solutes are separated, with the exception of crotononitriles and 3-butenenitrile. For these two solutes, the poor separation is probably connected with strong gas–solid adsorption in the column. They were also not separated at a higher temperature (180°C). The behaviour of this column with respect to the other nitriles is not optimized: C_2N_2, HCN, 2-methylpropanenitrile and butanenitrile do not give symmetrical peaks. With HCN, the retention time decreases markedly when the temperature increases up to *ca.* 150°C, then remains almost constant between 150 and 180°C. In addition, several peaks appear very large. The elution of all solutes requires at least *ca.* 30 min.

Fig. 2B shows an example of a chromatogram obtained for the same gaseous sample by programming the column temperature (isothermal at 100°C for 2 min, then increased at 25°C/min to 185°C). The separation of all solutes is accomplished in less than 12 min. The peaks of crotononitriles and 3-butenenitrile remain flat as before and are unsuitable for quantitative analysis of trace compounds. For the other nitriles, the chromatographic peaks have the same shape as previously.

This study has shown that the Al_2O_3–KCl PLOT column has a very high retention for nitriles and confirmed the general behaviour of this column relative to polar compounds. Nevertheless, C_1–C_4 nitriles are eluted from 10 m × 0.32 mm I.D. Al_2O_3–KCl PLOT column, but mainly with asymmetric and broad peaks.

Previous studies have demonstrated that a 50 m × 0.32 mm I.D. fused-silica Al_2O_3–KCl PLOT column is very effective for the separation of C_1–C_{10} hydrocarbons

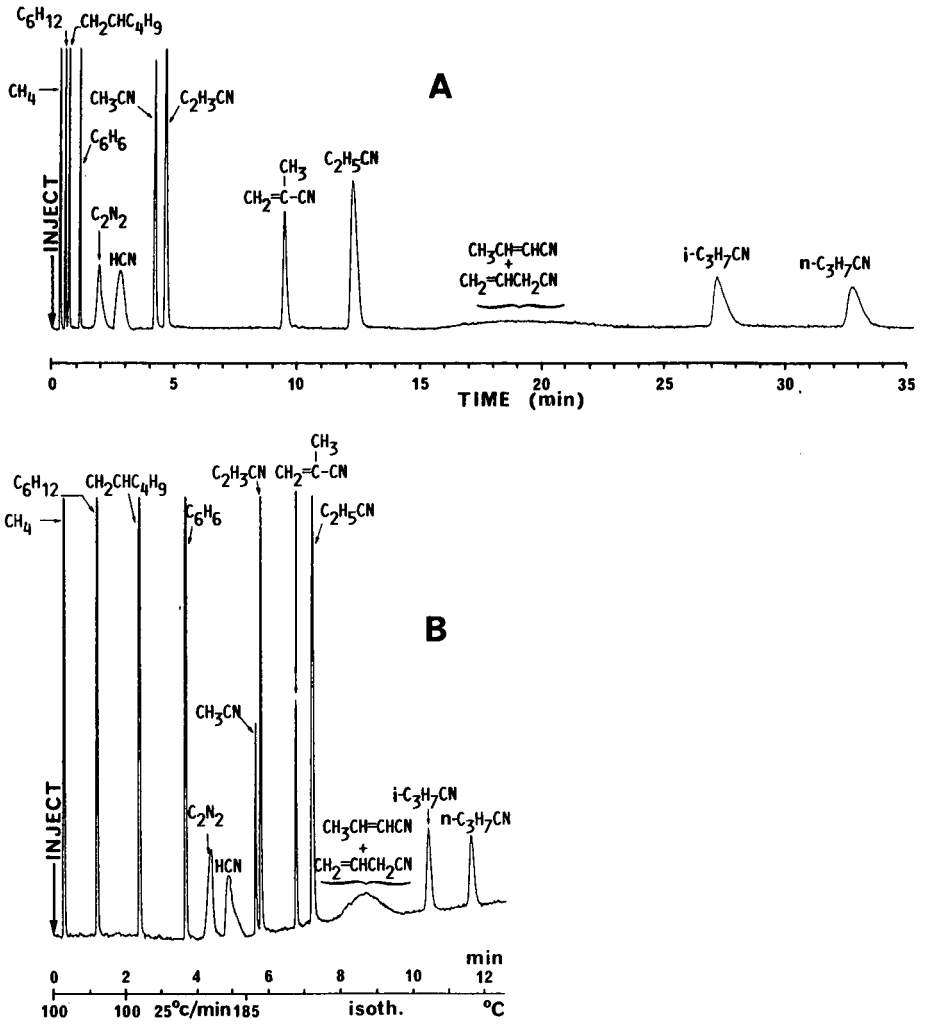

Fig. 2. Gas chromatogram of a gaseous mixture of methane, C_6 hydrocarbons (cyclohexane, 1-hexene and benzene) and C_1–C_4 nitriles, (A) at 150°C and (B) with temperature programming (2 min isothermal at 100°C, then increased at 25°C/min to 185°C). Carrier gas, hydrogen; outlet flow-rate, 4.4 ml/min; total amount injected, 10–30 nmol (hydrocarbons and C_1–C_4 nitriles), 50 nmol (C_2N_2) and 150 nmol (HCN); split mode (25:1); flame ionization detector.

at low temperatures[2–4]. Under these conditions nitriles are not eluted even after several hours. Hence the presence of nitriles does not prevent the use of this column for the rapid GC analysis of light hydrocarbons. In addition, it must be pointed out that no noticeable change in retention times was observed for the hydrocarbons studied after several injections of nitriles in relatively large amounts. Consequently, the adsorption of these polar compounds on the alumina surface does not seem to markedly alter its characteristics and does not change its chromatographic behaviour relative to the hydrocarbons. Such a column could be suitable for use in GC–mass

spectrometric experiments in the Cassini mission, specifically for the analysis of the hydrocarbons present in Titan's atmosphere.

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CHROM. 22 494

Note

Identification of monosaccharides by high-performance liquid chromatography using methanolysis and a light-scattering detector

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Structural studies of glycoconjugates rely heavily on chromatographic separations, which are of particular importance because of the great diversity of carbohydrates and large number of isomers. Before the separation of individual monosaccharides it is necessary to cleave glycosidic linkages of glycoconjugates by acid hydrolysis or methanolysis.

Acid hydrolysis of glycoconjugates followed by ion-exchange chromatography[1], high-performance liquid chromatography (HPLC)[2] with derivatization[3,4] or gas chromatography (GC)[5] has been extensively used. However, the conditions chosen for acid hydrolysis are usually a compromise between obtaining complete release of each sugar and preventing excessive destruction; liberated sugars differ in their stability to acids. Morever, separation usually requires derivatized samples.

Methanolysis offers the advantage of cleaving O-glycosidic linkages in a one-step procedure[6] and does not cause significant destruction of neutral and amino sugars[7]. Numerous analytical techniques using gas chromatography have been described[8,9], but all these methods require a derivatization of methyl glycosides and are often time consuming.

Various methods have been employed for the direct separation of methyl glycosides by HPLC[10,11], but analyses are limited by the detection of methyl glycosides. Some reports have appeared on the HPLC of methyl glycosides using mass spectrometric detection[12] or refractive index detection[10,13].

This paper describes a method for a rapid determination of neutral and amino sugars present in polysaccharides and glycoconjugates, involving methanolysis and HPLC analysis of the resulting methyl glycosides with a light-scattering detector. This method was applied successfully to the determination of monosaccharides of the well characterized glycoproteins α_1-acid glycoprotein and ovalbumin. It was also used for the determination of the sugar composition of polysaccharides such as gum arabic and gum karaya.

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EXPERIMENTAL

HPLC was carried out using a Perkin-Elmer L400 pump and a DDL 11 light-scattering detector (Cunow/Translab); data were collected on a Spectra-Physics SP4400 integrator. Nebulization of the eluent was provided by a stream of nitrogen at 29 p.s.i. The nebulized solvent was evaporated at 30 ± 1°C. The column was a Spherisorb ODS-2 (250 × 4.6 mm I.D.) (Societé Française de Colonnes Chromatographiques, SFCC). The samples were finally run at flow-rate of 0.5 ml/min with water–methanol (97:3) as the mobile phase. All solvents were of HPLC grade and were filtered through a 0.2-μm Millipore membrane; water was distilled.

Standard of monosaccharides, some of their methyl glycoside derivatives and ovalbumin were obtained from Sigma. Mesoinositol, used as an internal standard, was purchased from BDH Biochemicals. The α_1-acid glycoprotein was provided by Professor G. Durand (Laboratory of Biochemistry, Faculty of Pharmacy, Chatenay-Malabry, France) and gums were obtained from Iranex.

Methanolysis of the standard sugars, glycoproteins or polysaccharides was performed using the method of Zanetta *et al.*[9] with a few modifications. Methanolic hydrochloric acid (1.2 *M*) was diluted with anhydrous methanol to yield a concentration of 0.6 *M*. Samples containing glycoprotein or standard carbohydrate together with mesoinositol were dried over P_2O_5 under vacuum prior to methanolysis. Methanolysis was carried out by dissolving 100 μg of dried sugar or 500 μg of glycoprotein in 1 ml of the above solution in a sealed pyrex glass tube.

Samples were heated at 70°C for 20 h. Acid was removed under a stream of nitrogen at room temperature and the sample was dissolved in distilled water and injected into the chromatograph.

RESULTS AND DISCUSSION

Nine methyl glycosides, including neutral and amino sugars that commonly occur in glycoproteins and mesoinositol, often used as an internal standard in glycoprotein analysis[7], were separated on a Spherisorb ODS-2 column with water–methanol (97:3) (Fig. 1). Hexoses were eluted first, followed by pentoses, hexosamines, deoxyhexoses and uronic acids (Table I). Using a flow-rate of 0.5 ml/min the nine sugars were eluted within 16 min and detected using a light-scattering detector with an evaporation temperature of 30°C and a gas pressure of 29 p.s.i.

Various mobile phase compositions were studied in order to show the effect of adding an organic solvent to an aqueous eluent on chromatographic behaviour of methyl glycosides. Using water as eluent, the less polar methyl glycosides (deoxyhexoses) such as methyl rhamnoside and methyl fucoside were eluted after 21 min and the two methyl glycosides of fucose overlapped with the α-methyl glycoside of N-acetylglucosamine. Much better resolution was obtained by adding methanol to the eluent. We adopted 3% aqueous methanol because the derivatives of N-acetylglucosamine, fucose and rhamnose were partially separated with lower percentages of methanol, and with higher percentages of methanol the selectivity for the first eluted methyl glycosides was poor. Acetonitrile was also tested and gave the same elution profile except that a good separation required an acetonitrile content lower than 1%.

Methanolysis produces a mixture of methyl glycosides which do not reveal the

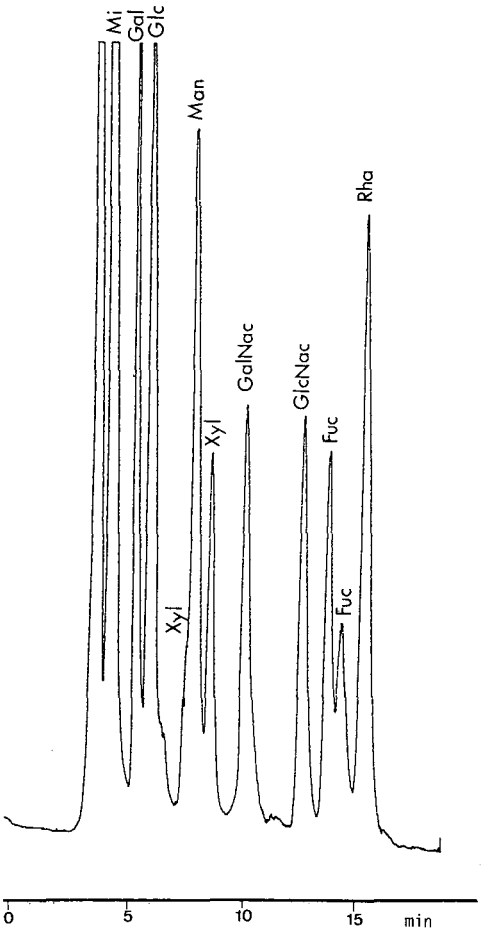

Fig. 1. Separation of a mixture of methanolysed monosaccharides on a 250 × 4.6 mm I.D. Spherisorb ODS-2 column at a flow-rate of 0.5 ml/min. Eluent, water–methanol (97:3); detector temperature, 30°C; pressure, 29 p.s.i.; photomultiplier gain, 9. Mi = Mesoinositol; Gal = galactose; Glc = glucose; Xyl = xylose; Man = mannose; GlcNac = N-acetylglucosamine; GalNac = N-acetylgalactosamine; Fuc = fucose; Rha = rhamnose.

anomeric configuration of the glycosidic linkage in polysaccharides or glycoproteins. The anomeric effect associated with a methoxy group causes an increase in the proportion of the anomer with an axial methoxy group on the more stable conformer of a methylpyranoside. Hence there is a higher proportion of α-anomer with galacto-, gluco-, manno- and xylo- configurations, as showed in previous studies[14]. Some of the isomers of monosaccharides were identified by comparison between the retention times obtained by methanolysis of a mixture of monosaccharides (Table I) (Fig. 1) and those obtained from standard methyl glycosides (Fig. 2). Methanolysis of individual sugars was used to determine whether or not some anomeric methylglycosides were formed and in what amounts; both galactose and mannose gave rise to a secondary anomer (less than 2%) which was eluted in the shoulder following the methyl glucoside

TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF METHYL GLYCOSIDES

Column, Spherisorb ODS-2; eluent, water–methanol (97:3); flow-rate, 0.5 ml/min.

Sugar	*Retention time (min)*	*Capacity factor*	*Anomer*[a]
Mesoinositol	5.04	0.22	–
D-Galactose	6.23	0.51	α-Pyranoside
D-Glucose	6.78	0.64	α-Pyranoside
D-Xylose	7.80	0.89	β-Pyranoside
	9.31	1.25	α-Pyranoside
L-Arabinose	7.93	0.92	–
D-Mannose	8.64	1.09	α-Pyranoside
N-Acetyl-D-galactosamine	10.84	1.62	–
N-Acetyl-D-glucosamine	13.24	2.20	α-Pyranoside
L-Fucose	14.65	2.55	α-Pyranoside
	15.03	2.64	–
L-Rhamnose	15.99	2.87	α-Pyranoside
D-Galacturonic acid	16.75	3.05	–

[a] The configuration of isomers was defined using standard methyl glycosides.

peak (retention times 6.81 and 6.97 min, respectively). However, this is of minor importance as the heights of the main peaks can be readily measured without interference from other sugars. Under the conditions of trimethylsilyl ether formation, used in GC, sugars give rise to a mixture of α- and β-anomers and when dealing with a mixture overlap is inevitable. Using our method, all the sugars except fucose and xylose give rise to only a main peak and it is therefore more suitable for quantitative analysis.

The influence of some parameters of the evaporative light-scattering detector on the response factor was investigated. Fig. 3 shows a plot of detector response *versus* the atomiser inlet nitrogen pressure between 17 and 34 p.s.i. The curves showed no trend with increasing temperature. This phenomenon was previously observed with pyrene by Righezza and Guiochon[15], who noticed that when the solvent flow-rate was below 0.7 ml/min the response did not vary significantly with the gas flow-rate.

The effect of the evaporator temperature setting on the detector response was investigated for several methyl glycosides. We observed a similar behaviour towards increasing temperature for similar sugars (Fig. 3); neutral methyl glycosides such as methyl glucopyranoside and methyl galactopyranoside showed nearly a 50% decrease in response at elevated temperature, whereas methyl glycosides of deoxyhexoses such as fucose and rhamnose showed a rapid decline with a very small temperature increase. In contrast, amino sugar derivatives and mesoinositol showed a slight decrease. In previous studies with other solutes[16,17] the various decreases in detector response with increasing temperature were attributed to a decrease in particule size. Methyl fucopyranoside and methyl rhamnopyranoside are relatively volatile, hence the decrease in response at elevated temperature is greater because it is also due to partial volatilization; above 38°C these derivatives are hardly detectable. The noise level was

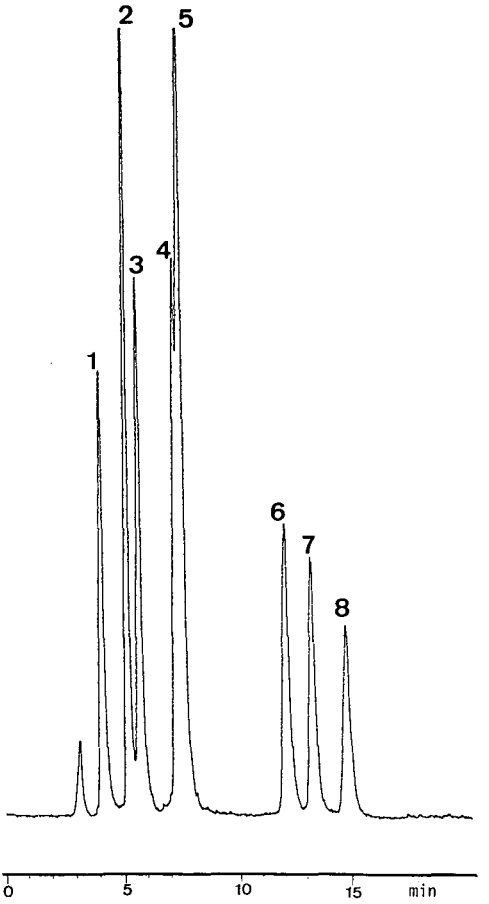

Fig. 2. Separation of a mixture containing standard methyl glycosides (conditions as in Fig. 1). Peaks: 1 = mesoinositol; 2 = methyl-α-D-galactopyranoside; 3 = methyl-α-D-glucopyranoside; 4 = methyl--β-D-xylopyranoside; 5 = methyl-α-D-mannopyranoside; 6 = methyl-α-D-N-acetylglucosamine; 7 = methyl-α-L-fucopyranoside; 8 = methyl-α-L-rhamnopyranoside.

found to be dependent on the evaporation temperature, so it was necessary to work above 28°C to allow an acceptable noise level. The temperature had to be low enough to avoid solute vaporization and high enough to ensure complete vaporization of the solvents; the optimum temperature for analysing the mixture of methyl glycosides was 30°C. As the latent heat of vaporization of water is very important[18], we chose a low gas velocity in order to permit complete vaporization of the solvent.

Detection limits, expressed as twice the baseline noise, were less than 30 ng for most methyl glycosides (methyl glucoside, methyl galactoside and methyl mannoside) and for mesoinositol, whereas for the other compounds they ranged from 50 to 100 ng. This method is at least as sensitive as some other methods using HPLC of methyl glycosides[13] or derivatized methyl glycosides[19].

The technique was applied to the determination of monosaccharides of two glycoproteins, α-GPA, with a high content of sugar (40%, w/w), and ovalbumin, which

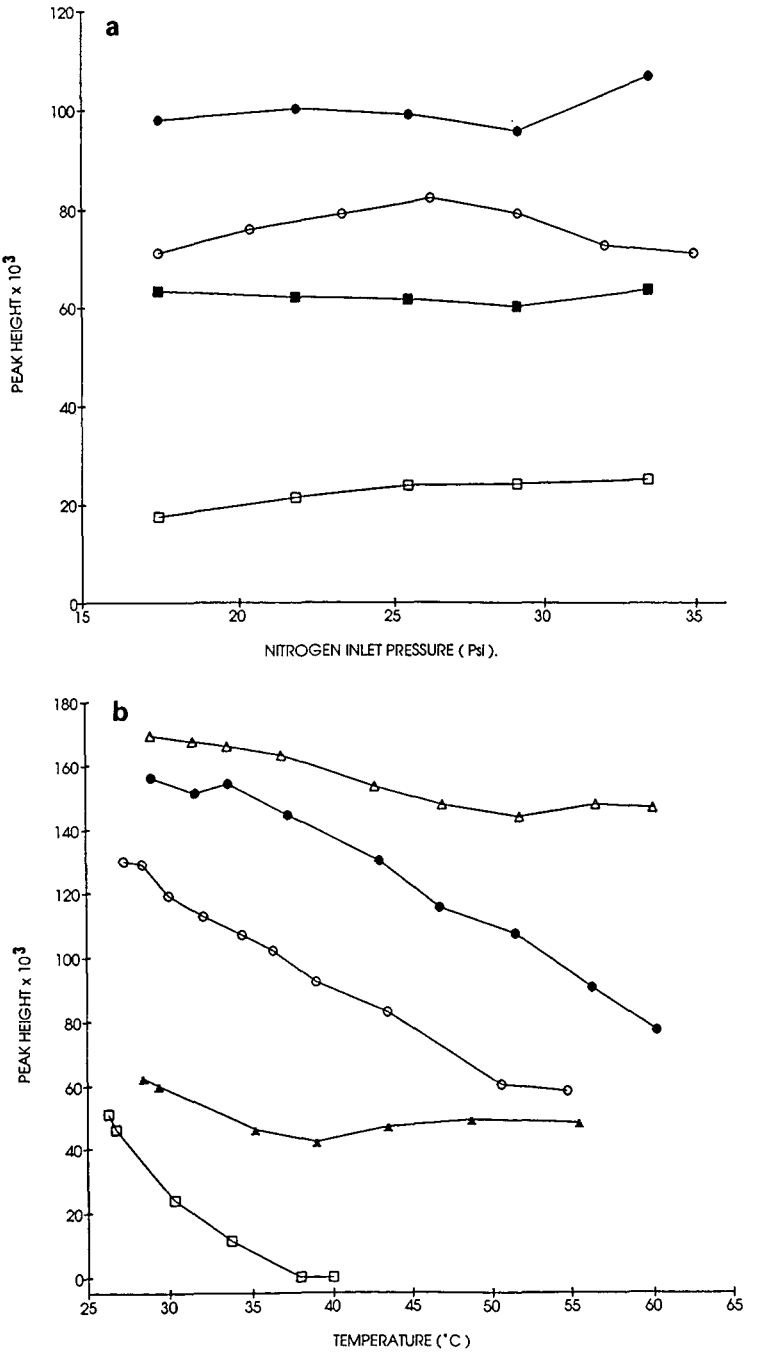

Fig. 3. Effect of (a) nitrogen inlet pressure and (b) temperature on the detector response for mesoinositol and several methyl glycosides. ● = Methyl-α-D-glucopyranoside; ○ = methyl-α-D-galactopyranoside; ■ = methyl-α-D-mannopyranosides;□ = methyl-α-L-fucopyranoside; ▲ = methyl-α-D-N-acetylglucosamine; △ = mesoinositol. Values are averages of three successive measurements for each temperature or each pressure in terms of peak height.

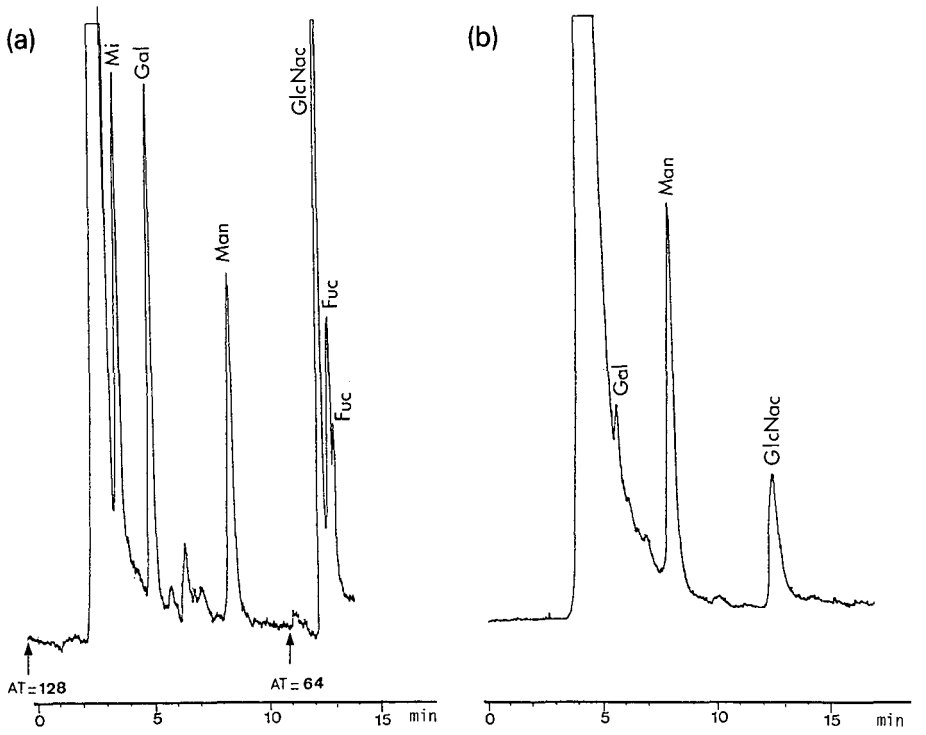

Fig. 4. Analyses of (a) α_1-acid glycoprotein and (b) ovalbumin. Conditions as in Fig. 1 with photomultipier at gain 10. AT = Attenuation.

contains less carbohydrate (5%, w/w). Two typical chromatograms are shown in Fig. 4. α-GPA contained mannose, galactose, N-acetylglucosamine and fucose, whereas methanolysis of ovalbumin gave rise mainly to mannose, some N-acetylglucosamine and traces of galactose. The method was also applied to the determination of the monosaccharide constituents of two polysaccharides. The chromatograms obtained from gum arabic and gum karaya are shown in Fig. 5.

Quantitative analysis was carried out. As expected from previous studies on other solutes[18,20], the calibration graphs obtained with four methyl glycosides showed a sigmoidal response of the detector in the concentration range studied (1–15 mg per 100 ml) (Fig. 6). Plots of peak height *versus* sample concentration in double logarithmic coordinates are linear (Fig. 7) with a similar slope for all the solutes of 1.277 ± 5.9%, indicating a similar mode of response. The regression data are given in Table II. The data obtained from three quantitative analysis of ovalbumin were in agreement with the literature values[4,8] as we obtained 23.8 and 21.5 μg per mg of glycoprotein for mannose and N-acetylglucosamine, respectively (with relative standard deviations less than 5%). Methanolysis of a non-glycosylated protein, bovine serum albumin, was performed and showed no interference in the sugar chromatogram.

The method described is very simple as it requires only a single-step metha-

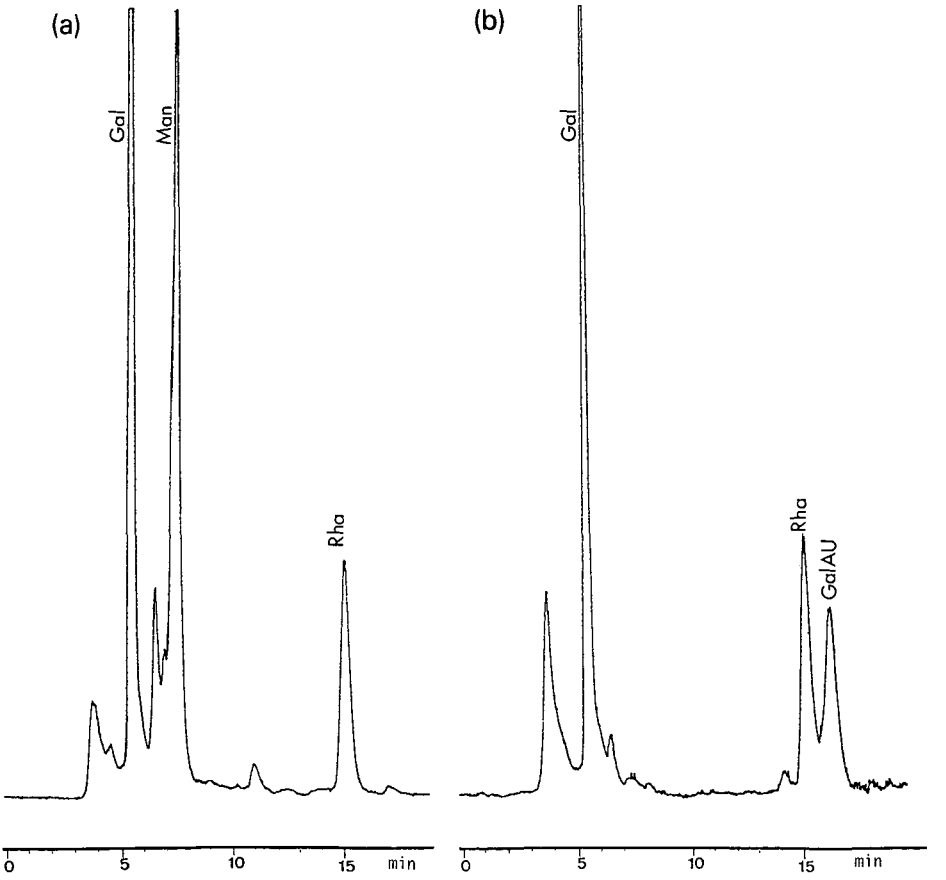

Fig. 5. Analyses of (a) gum karaya (b) gum arabic. Conditions as in Fig. 4. GalAU = galacturonic acid.

nolysis at 70°C followed by a chromatographic separation of the derivatives within 16 min. All carbohydrate moieties, including N-acetylamino sugars, contained in glycoproteins can be determined from a single hydrolysate. Moreover, the method can be applied to a wide range of glycoconjugates, including polysaccharides. This analysis

TABLE II

REGRESSION DATA FOR LOGARITHMIC PLOTS OF DETECTOR RESPONSE AGAINST CONCENTRATION OF METHYL GLYCOSIDES

Methyl glycoside	*Correlation coefficient*	*Slope*	*Intercept*
D-Galactose	0.997	1.273	4.54
D-Mannose	0.994	1.251	4.27
N-Acetyl-D-Glucosamine	0.997	1.203	4.08
L-Fucose	0.994	1.380	3.71

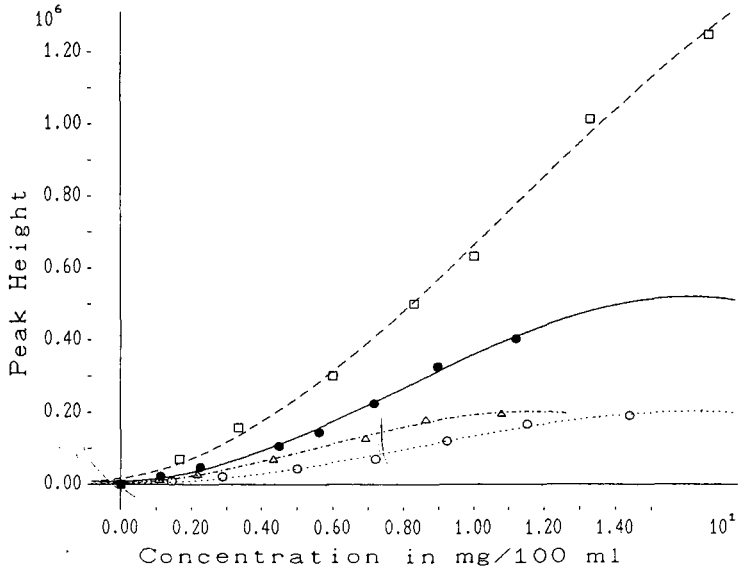

Fig. 6. Calibration graphs with the light-scattering detector for four methylglycosides. Values are means of three consecutives injections. □ = Methyl-α-D-galactopyranoside; ● = methyl-α-D-mannopyranoside; ○ = methyl-α-L-fucopyranoside; △ = methyl-α-D-N-acetylglucosamine.

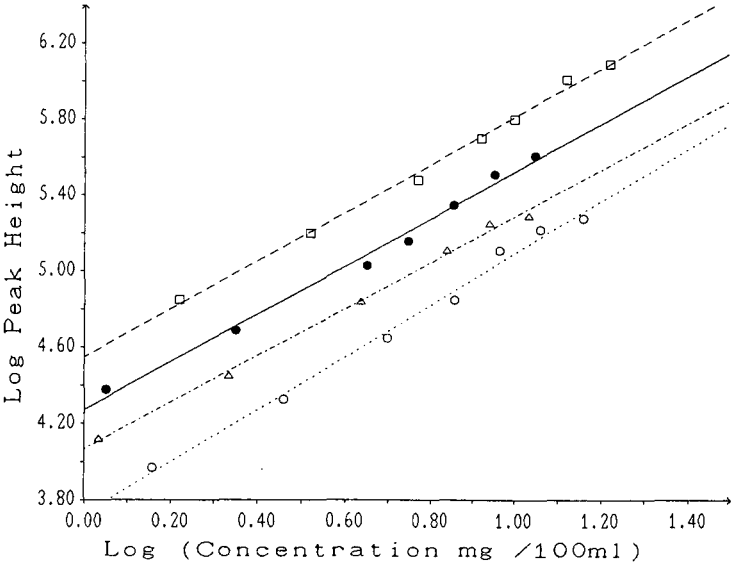

Fig. 7. Log–log plot of the light-scattering detector response against concentration of methyl glycoside. □ = Methyl-α-D-galactopyranoside; ● = methyl-α-D-mannopyranosides; ○ = methyl-α-L-fucopyranoside; △ = methyl-α-D-N-acetylglucosamine.

is interesting because of the main chromatographic peak obtained for most compounds. The linear response of the detector obtained in double logarithmic coordinates permitted quantitative analysis.

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CHROM. 22 479

Note

High-performance liquid chromatography of thiazolidinic compounds

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Thiazolidinic derivatives are formed by condensation of aminothiols with aliphatic or aromatic compounds containing a CHO group[1,2]: reactions of pyridoxal-5'-phosphate (PLP) and pyridoxal (PL) with L-cysteine, L-cysteine ethyl ester and homocysteine have been described[3–8].

Aromatic thiazolidinic compounds (TA) could have physiological importance; in fact, several workers have reported the reaction between free aminothiols and enzyme-bound PLP[9–13]; they noted that the TA have no great affinity for the apoenzyme, and are quickly released (this treatment is frequently used to resolve the coenzymic PLP form)[10–14]. Moreover, the synthesis of these derivatives occurs easily, under very mild conditions, (37°C, pH 7, low concentrations) and hence it could occur spontaneously in the cell.

Up to now, the absence of a reliable method has precluded the study of the possible metabolism of TA *in vivo*. The spectrophotometric procedure of Guidotti *et al.*[15] is very useful for the determination of the aliphatic TA but in to our experience it is unsuitable for aromatic derivatives. Recently other workers have published different high-performance liquid chromatographic (HPLC) procedures for the separation of thiazolidinic derivatives. Sen *et al.*[16] report the separation of some compounds found in fried bacon and in other types of meat. Van Doorn *et al.*[17] and, more recently, Ogata and Taguchi[18] have developed HPLC methods for the determination of urinary 2-thiothiazolidine-4-carboxylic acid as an index of carbon disulphide exposure. Their working conditions and thiazolidinic derivatives are very different from ours.

Here we describe two different procedures for the determination of TA obtained by condensation between PLP and L-cysteine (TAL) or D-cysteine (TAD): a spectrophotometric assay using a simple variation of Ellman's reaction[19] and a new HPLC procedure.

EXPERIMENTAL

Chemicals

PLP, L- and D-cysteine, potassium dihydrogenphosphate and potassium hydro-

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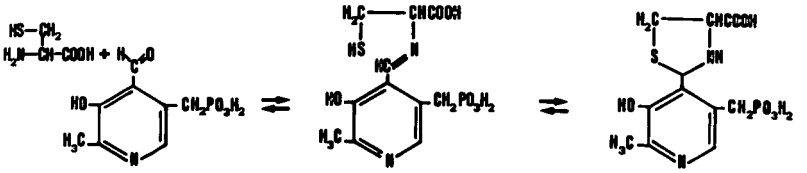

Fig. 1. Formation of thiazolidin-4-carboxylic acid.

genphosphate were obtained from Merck (Darmstadt, F.R.G.). 5',5'-Dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman's reagent) and Norit A were purchased from Sigma (St. Louis, MO, U.S.A.). Methanol (HPLC-grade) was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.).

Preparation of thiazolidin-2-(2'-methyl-3'-hydroxy-4'-pyridyl-5'-methylsulphonic)carboxylic acid

This compound was synthesized from PLP and either L- or D-cysteine according to Angeletti and Haertelt[6] (Fig. 1). The compound showed a specific UV spectrum with a absorption maximum at 330 nm ($\varepsilon = 6.41\,\text{mmol}^{-1}\,\text{cm}^{-1}$). Spectra were obtained with a Shimadzu (Kyoto, Japan) UV-160 spectrophotometer.

Application of Ellman's reaction

The determination of TA was carried out via Ellman's reaction (Fig. 2).

In solution the closed form of TA is in equilibrium with the open form (see Fig. 1) and its free SH groups react with DTNB; the equilibrium is totally shifted toward the formation of Ellman's derivative, which has an absorption maximum at 412 nm. The reaction of TA with DTNB is slower than the reaction of the same reagent with L-cysteine, which is almost istantaneous. In a mixture of L-cysteine and TA, the absorbance at 412 nm (A_{412}) at 0 min gives the amount of L-cysteine present; A_{412} after 40 min is due to TA and L-cysteine, so the TA content is derived from the value of ΔA_{412}.

The reaction was carried out as follows: 70 μl of 10 m*M* DTNB were added to 10 ml of 0.03 m*M* thiazolidin-4'-carboxylic acid in 50 m*M* potassium phosphate buffer (pH 7.5) and readings were immediately taken at 412 nm. After 40 min at 25°C, readings were again taken at the same wavelength.

Preparation and utilization of rat liver supernatant

Rat liver supernatant prepared as shown in Fig. 3 was used. A 2-μmol amount of

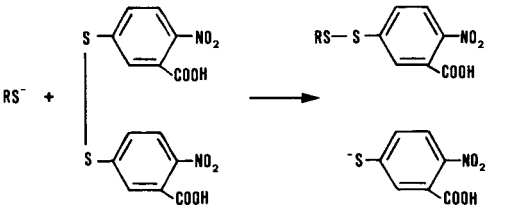

Fig. 2. Formation of 3-carboxy-4-nitrothiophenol derivative.

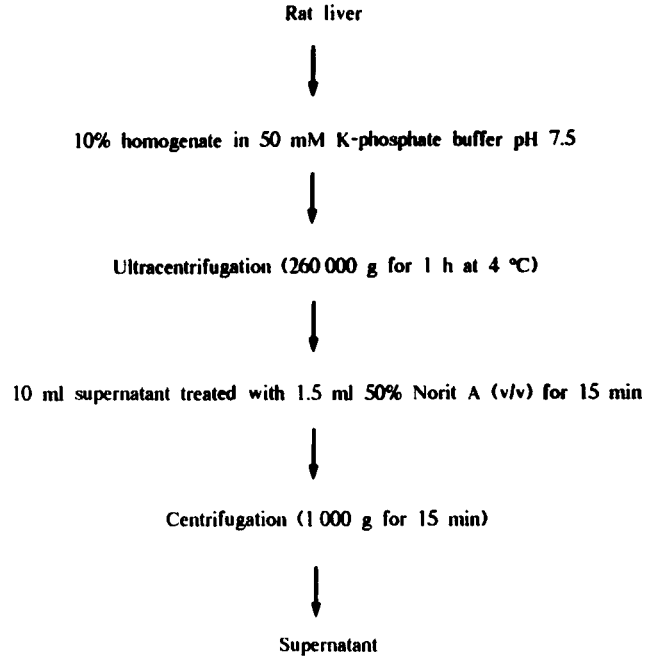

Fig. 3. Preparation of rat liver supernatant.

the thiazolidinic compound was added to 0.5 ml of supernatant, immediately deproteinized with 2 *M* hydrochloric acid (0.5 *M* final concentration), centrifuged at 8000 × *g* and diluted with 50 m*M* potassium phosphate buffer (pH 7.5) until the TA reached final concentration of 0.1 m*M*. The blank was obtained by replacement of the supernatant with 0.5 ml of the same buffer. A 20-μl volume of this solution (2 nmol) was injected into the HPLC system.

Apparatus and chromatographic conditions

A Vista 5500 high-performance liquid chromatograph (Varian, Sunnyvale, CA, U.S.A.) equipped with a variable-wavelength UV detector (Model 2550, Varian) and an electronic integrator (Model 4290, Varian) were used. A ready-to-use prepacked column (250 × 4.6 mm I.D.) of Supelcosil LC-18, 5 μm (Supelco, Bellefonte, PA, U.S.A.), protected by a precolumn (20 × 4.6 mm I.D.) filled with the same packing (Supelguard, Supelco) completed the analytical system.

The mobile phase was 0.01 *M* potassium phosphate buffer (adjusted to pH 5.5 with 0.5 *M* potassium hydroxide)–methanol (95:5, v/v) at flow-rate of 1 ml/min. Detection was performed at 254 nm.

RESULTS AND DISCUSSION

Optimum conditions

A preliminary study involving the separation of cysteine, TA and PLP led us to

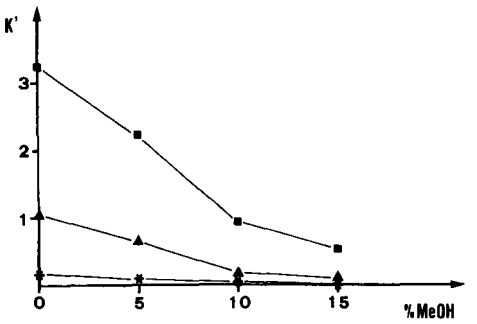

Fig. 4. Effect of methanol concentration on retention (✱) cysteine, (▲) TA and (■) PLP (MeOH = methanol.

consider the effect of different concentrations of organic solvent in the mobile phase (Fig. 4). Fig. 5 shows that a good separation is obtained with isocratic elution.

Cysteine was detected at 204 nm as it has no characteristic peak in the UV spectrum, either in water or in 10 mM potassium phosphate buffer (pH 5.5), but shows a non-specific absorption at low wavelengths (molar absorptivity 0.016 l mmol^{-1} cm^{-1} at 254 nm and 1.027 l mmol^{-1} cm^{-1} at 204 nm) (Fig. 5). The peak eluted at 3.24 min was also submitted to the Ellman reaction, giving a positive result. In subsequent experiments we used detection at 254 nm to minimize the non-specific interferences of buffer and samples at low wavelengths.

Good linearity was obtained for all amounts of TA and PLP used (0.1–20 nmol). The correlation coefficients for TA and PLP were 0.995 ($p < 0.01$) and 0.999 ($p < 0.01$), respectively, and the regression equations of the calibration graphs were $A = 157.52\ C + 33.86$ and $A = 218.85\ C - 11.56$, respectively, where A = peak area and C (nmol) = amounts of reagent.

The overall between-run and between-day precisions of the retention times and peak areas were studied and the results are presented in Table I.

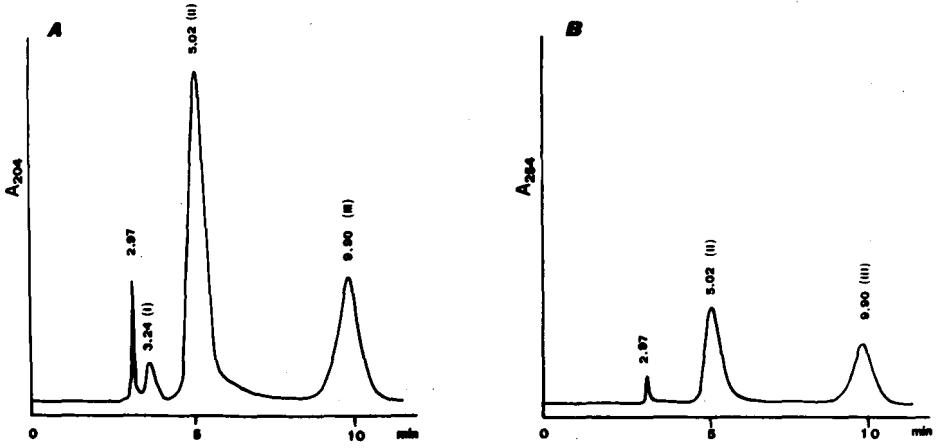

Fig. 5. Separation of (I) cysteine, (II) TA and (III) PLP with detection at (A) 204 and (B) 254 nm. Injection volume: 20 μl of solution 1 mM in each standard.

TABLE I

REPRODUCIBILITY AND ACCURACY OF RETENTION TIMES AND PEAK AREAS OF TA AND PLP

Parameter	*Compound*	*Retention time (min)*	*S.D. (n=5)*	*Relative S.D. (%)*	*Peak area (arbitrary units)*	*S.D. (n=5)*	*Relative S.D. (%)*
Between-run precision (within 1 day)	TA	4.99	0.03	0.60	3202.5	68.3	0.21
	PLP	10.2	0.17	1.66	4297.4	45.0	1.04
Between-day precision (7 days)	TA	4.99	0.02	0.40	3236.2	48.5	1.50
	PLP	10.21	0.04	0.39	4346.1	44.2	1.02

Effect of addition of rat liver supernatant to TA solution

We wanted to ascertain whether the TAL was modified in the presence of rat liver supernatant. The supernatant was added to TAL, as described under Experimental. The elution patterns were not much influenced by the addition of supernatant, as shown in Fig. 6, where the area of peak I in B is more than 90% of peak I in A. The same experiment was repeated for different final concentrations of TAL (2, 1.5 and 0.5 m*M*), giving good proportionality and recovery.

TAD was also measured under all of the above conditions. The compound showed the same retention time as TAL and the same behaviour in both the absence and presence of rat liver supernatant.

Ellman's reaction

The thiazolidinic compound was submitted to the Ellman reaction: suitable aliquots were treated as indicated under Experimental and readings were taken at different times (0 and 40 min); the colour was stable after 40 min. Linearity

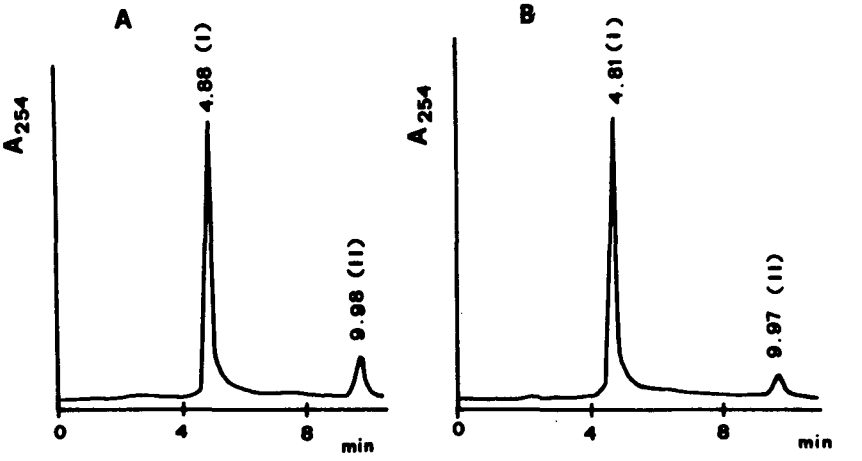

Fig. 6. Chromatography of TAL in the presence of tissue extracts: 2 nmol of (I) TAL in (A) the absence and (B) the presence of rat liver supernatant; (II) PLP derived by spontaneous decomposition of TAL.

(absorbance *versus* concentration) was tested on standards in buffered solutions and the regression equation of the calibration graph was $A_{412} = 0.02\,C + 0.025$, $r = 0.998$, $p < 0.01$. The linearity was excellent between $C = 6$ and 30 nmol.

The spectrophotometric procedure showed a low sensitivity (more than 6 nmol) and long times of execution (40 min), and it could not be applied to the TA compounds in presence of rat liver supernatant owing to severe tissue interference (not yet analysed).

From the results it is evident that the thiazolidinic derivatives of both L- and D-cysteine, can be easily separated from PLP by HPLC. The procedure can easily be applied to tissue extracts, and in this way it should be possible to ascertain whether, and in which tissue or organisms, TA compounds are either synthesized or degraded.

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Note

Separation of bleomycins and their deamido metabolites by high-performance cation-exchange chromatography

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Bleomycin (BLM), a mixture of basic glycopeptides isolated from *Streptomyces verticillus*, is widely used as an antitumor antibiotic in the treatment of testicular carcinomas and lymphomas[1]. The clinical BLM mixture (Blenoxane) contains at least eleven different BLMs, although the two principal components are BLM A_2 (55–70%) and BLM B_2 (25–30%) (Fig. 1). It has been suggested that *in vivo* antitumor activity of these two compounds as well as their toxicity to non-malignant tissues is limited by the enzyme bleomycin hydrolase, which we have recently purified to homogeneity and characterized by fast protein liquid chromatography (FPLC)[2,3]. This enzyme generates the inactive metabolites, deamido BLM A_2 (BLM dA_2) and deamido BLM B_2 (BLM dB_2) from BLM A_2 and BLM B_2, respectively[4–7]. Characterization of these BLM metabolites has been greatly restricted due to the lack of a general method for their isolation in pure forms. Furthermore, the conventional method for isolation of components of BLM mixture is CM-Sephadex column chromatography, which is both laborious and time consuming[8–10]. In this paper, we describe the use of FPLC for the rapid separation of BLM dA_2 from BLM A_2 and BLM dB_2 from BLM B_2 as well as for the separation of BLM A_2 and BLM B_2 from the clinical BLM mixture.

EXPERIMENTAL

Reagents and materials

Clinical BLM mixture (Blenoxane) was a gift from Bristol-Myers Squibb (Wallingford, CT, U.S.A.). BLM A_2 and BLM B_2 were purified as previously described[6,11]. Enzymatically-generated, authentic BLM dA_2 and BLM dB_2 standards were gifts from Nippon Kayaku (Tokyo, Japan). Additional BLM dA_2 and BLM dB_2 were enzymatically prepared as described previously[6]. Ammonium formate was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Chromatographic equipment and conditions

The FPLC system (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) used in the present studies consisted of an LCC-500 gradient programmer controlling

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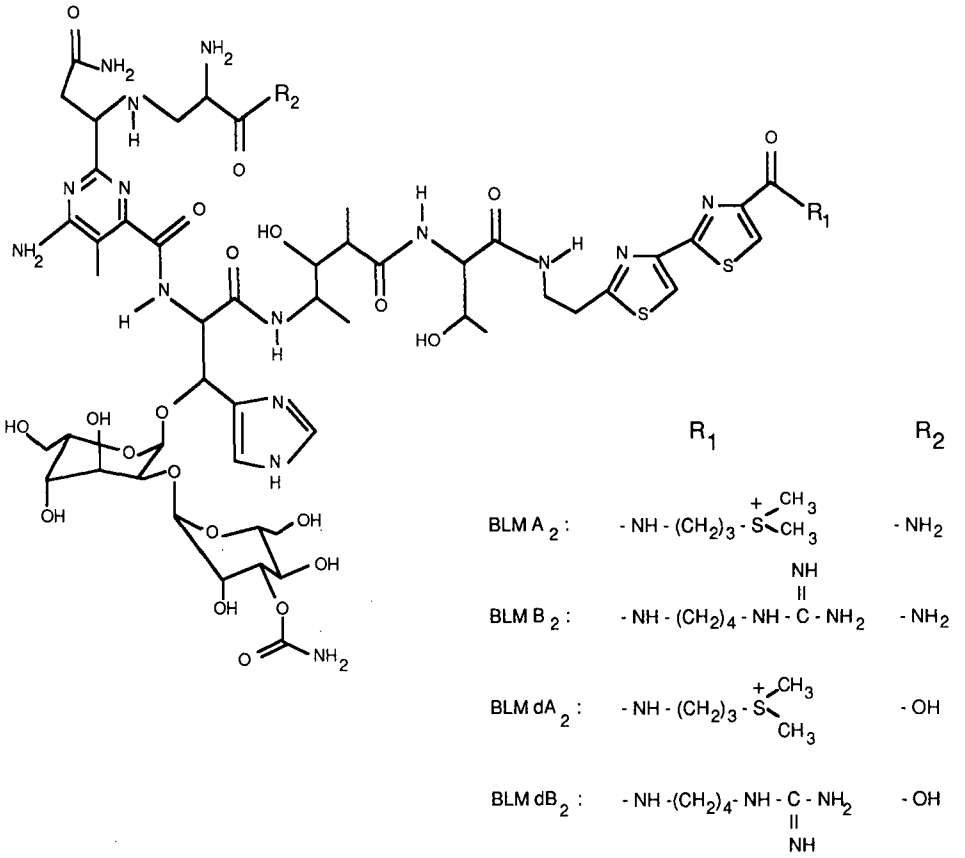

Fig. 1. Structures of major BLM components and their deamido metabolites.

two P-500 reciprocating pumps, a mixing chamber, an MV-7 injector valve for introduction of sample, a Mono S HR 5/5 cation-exchange column (5 cm × 5 mm I.D., 10 μm particle size, 25 000 theoretical plates/m) and a 280-nm fixed-wavelength single-path UV monitor coupled to a single-channel Kipp & Zonen (The Netherlands) pen recorder. Fractions were collected with a FRAC-100 fraction collector (Pharmacia). The eluting buffers were: buffer A, 0.05 *M* ammonium formate (pH 6.5); buffer B, 1.0 *M* ammonium formate (pH 6.5). Appropriate gradients and flow-rates were chosen for each separation. For each separation method used, reproducible elution profiles were obtained. All chromatographic separations were carried out at 4°C. Buffer solutions were filtered through a 0.2-μm filter and degassed prior to use. All samples were also filtered before injecting onto the column.

RESULTS AND DISCUSSION

The use of ion-exchange chromatography as a modern biochemical separation technique has made it possible to isolate and characterize many peptides and proteins

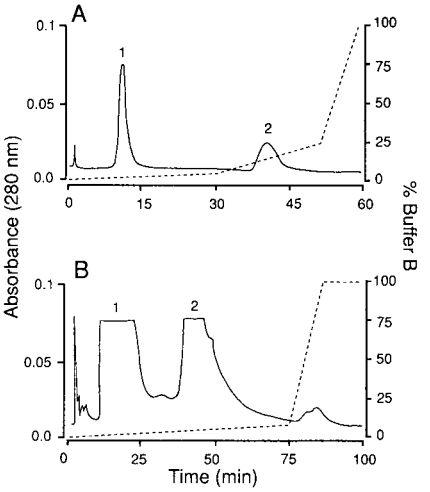

Fig. 2. Mono S (HR 5/5) cation-exchange chromatographic separation of (A) BLM A_2 and BLM B_2 from 10 μg of a clinical BLM mixture; elution was carried out with a gradient of 2–5% buffer B over 30 min, 5–25% buffer B from 30–50 min and 25–100% buffer B from 50–60 min; flow-rate = 1.0 ml/min; (B) BLM A_2, BLM B_2 and other minor BLM components from 1 mg of a clinical BLM mixture using gradients of 0–10% buffer B over 75 min and 10–100% buffer B from 75–85 min and a flow-rate of 1.0 ml/min. 1 = BLM A_2; 2 = BLM B_2. Solid lines, absorbance; dashed lines, percentage buffer B.

of biological importance. The antineoplastic agent BLM can be separated into its glycopeptide components, using CM-Sephadex chromatography[8–10]. Unfortunately, when conventional cellulosic ion-exchangers are used, the separation process is very lengthy. Polymer-based resins such as Mono S, Mono Q and Mono P have been used in place of conventional ion exchangers or chromatofocussing materials, because they possess high mechanical strength, are homogeneous in size and are stable over a wide pH range. Thus, we have examined the utility of Mono S cation-exchange resin to separate BLM components because of its superior resolving power and speed. In addition, we have used this methodology to address the more difficult problem of separating the known BLM metabolites.

Fig. 2A shows a gradient elution profile after cation-exchange chromatographic separation of BLM A_2 and BLM B_2 from clinical BLM mixture (Blenoxane). The clinical BLM (10 μg) was adsorbed to the cation-exchange column in the starting buffer (buffer A) and elution was carried out at a flow-rate of 1.0 ml/min, with a gradient of 2–5% buffer B over 30 min, 5–25% buffer B from 30–50 min and 25–100% buffer B from 50–60 min. As illustrated in Fig. 2A, BLM A_2 eluted first (retention time *ca.* 11 min). Due to strong electrostatic interactions between the basic guanidinium moiety of BLM B_2 and the strongly acidic SO_3^- groups on the Mono S beads, BLM B_2 eluted very slowly (retention time *ca* 40 min).

We have successfully extended the use of the analytical (HR 5/5) Mono S column to separate individual BLM components from quantities as large as 17.6 mg of the clinical BLM mixture. Fig. 2B illustrates the appearance and separation of other minor BLM components apart from BLM A_2 and BLM B_2, when 1 mg of BLM mixture

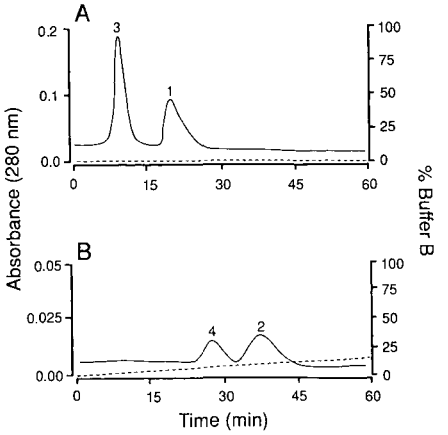

Fig. 3. Mono S (HR 5/5) cation-exchange chromatographic separation of the deamido BLM metabolites. (A) Separation of BLM dA_2 and BLM A_2, using buffer A and a flow-rate of 0.9 ml/min; (B) separation of BLM dB_2 and BLM B_2, using a gradient of 0–15% buffer B over 60 min and a flow-rate of 1.0 ml/min. 1 = BLM A_2; 2 = BLM B_2; 3 = BLM dA_2; 4 = BLM dB_2. Solid lines, absorbance; dashed lines, percentage buffer B.

(Blenoxane) was loaded onto the column. Elution was carried out at a flow-rate of 1.0 ml/min, with a linear gradient of 0–10% buffer B over 75 min followed by a gradient of 10–100% buffer B at the end of 85 min. Various fractions containing BLM A_2 and BLM B_2 were collected and lyophilized. The amount of BLM A_2 and BLM B_2 obtained was 0.55 mg and 0.235 mg, respectively, (based on absorbance at λ_{max} = 292 nm); this is in good agreement with previously calculated ratios of BLM A_2 and BLM B_2 found in the clinical mixture of BLM[1,10]. Assuming that *ca.* 90% of the mixture consisted of BLM A_2 and BLM B_2, the combined recovery of the two components was 87%. BLM A_2 and BLM B_2 from the clinical mixture eluted at the same relative positions as authentic BLM A_2 and BLM B_2 standards. The identity of BLM A_2 and BLM B_2 that eluted from the Mono S column was further confirmed by analysing the corresponding fractions by reversed-phase HPLC as described previously[12].

We then used Mono S column chromatography to separate the BLMs from their deamido metabolites, which were generated by the enzyme bleomycin hydrolase. A mixture of BLM A_2 and BLM dA_2 (total 25 μg) was injected onto a Mono S column and eluted at a flow-rate of 0.9 ml/min with 0.05 *M* ammonium formate. As shown in Fig. 3A, BLM dA_2 eluted first (retention time *ca.* 9 min) followed by BLM A_2 (retention time *ca.* 20 min). The metabolic cleavage of the amide bond in the β-aminoalanine moiety of BLM A_2 by the enzyme bleomycin hydrolase, produces BLM dA_2, a molecule with a lesser positive charge than the parent BLM A_2. Therefore, BLM dA_2 binds less to the Mono S column and elutes first. The identity of BLM dA_2 was also confirmed by reversed-phase HPLC as described previously[12]. Recovery of the two compounds was 90%.

Similar results were obtained with a mixture of BLM B_2 and its inactive metabolite BLM dB_2. Fig. 3B demonstrates a Mono S cation-exchange chromatographic separation of a mixture of BLM dB_2 and BLM B_2 (total 7 μg). A flow-rate of 1.0 ml/min and a linear gradient of 0–15% buffer B over 60 min were found to be

optimum for the separation since a steeper gradient did not resolve the compounds and a slower gradient resulted in very wide peaks on the chromatogram. Retention times of BLM dB_2 and BLM B_2 were found to be *ca.* 28 min and *ca.* 38 min, respectively. Confirmation of the identity for the separated components was provided by comparison with the migration of the authentic standards.

The chromatographic method described in this report can have broader biological applications beyond the separation of the BLMs. For example, a number of endogeneous bioactive peptides such as enkephalins, substance P and thyrotropin-releasing hormone contain terminal carboxamide moieties, which are essential for their biological activity[13]. Separation of the deamidated peptides from the carboxamide-containing peptides can be problematic. Therefore, it is possible that the Mono S/FPLC system could be generally useful for separating inactive deamido metabolites from other bioactive peptides that contain the protective carboxamide moieties[13].

In conclusion, the Mono S/FPLC provides a rapid method to separate individual components of BLM. The Mono S column chromatography also allows for the rapid separation of the deamido metabolite of BLM from the parent compound. Although we have separated relatively small amounts of BLM dA_2 and BLM dB_2 due to their limited availability, the existence of preparative columns should permit even greater amounts to be isolated. In contrast to ion-pair reversed-phase HPLC methods, the FPLC approach provides for a BLM that is free of contaminating organic acids or ion-pair agent, which are difficult to remove[14–16]. The use of ammonium formate as a buffer system in the FPLC method is advantageous because the salt can be easily removed from the separated compounds upon lyophilization. When BLM is complexed to Cu(II) the resolution is improved further. This versatile method should now allow for a more detailed characterization of the metabolites of an important class of biologically active glycopeptides.

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Note

Chiral sulphonated phosphines

IV[a]. High-performance liquid chromatographic separation of sulphonated phosphines

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There has been increasing interest during the last decade in the use of a two-phase system (water–organic solvent) in homogeneous organometallic catalysis where the catalyst is in the aqueous phase by the use of water-soluble ligands[1,2]. The water solubilization of phosphines is usually achieved by the introduction of a highly polar functional group such as an amine, carboxylic acid, hydroxide or sulphonate. Rhodium complexes of sulphonated phosphines are effective as catalysts in industry for hydroformylation[3,4] and in the synthesis of geranylacetone[5,6]. We have shown recently that asymmetric hydrogenation occurs in a two-phase system using chiral sulphonated phosphines[7–9], and that the enantioselectivity seems to depend on the degree of sulphonation of the chiral diphosphine. Hence it would be desirable to design a technique for the separation of these sulphonated phosphines, thus giving the composition of the sulphonated ligands used in hydrogenation.

As various aromatic sulphonic acids have been separated using reversed-phase ion-pair chromatography with a Hypersil SAS silica (C_1 or C_8) as the stationary phase, a quaternary ammonium salt (usually cetrimide) as the counter ion and water–propanol as the eluent[10–12], we thought that this technique, called "soap chromatography", would be the most appropriate in our case[13].

EXPERIMENTAL

Sulphonated phosphines (Fig. 1) were obtained as described previously[8]. Hexadecyltrimethylammonium bromide (cetrimide), tetrabutylammonium bromide (TBA), tetraethylammonium bromide (TEA) and tetramethylammonium bromide (TMA) were obtained from Fluka.

[a] For Part III, see ref. 9.

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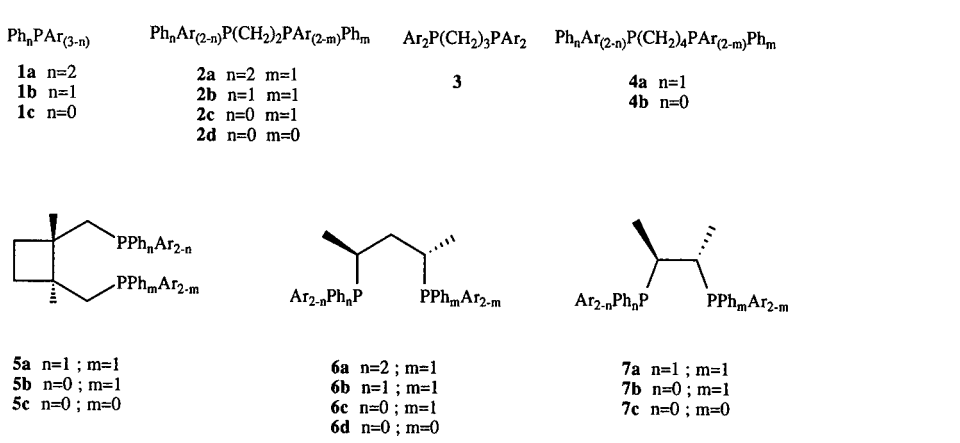

Fig. 1. Sulphonated phosphines. Ar = *m*-$C_6H_4SO_3Na$; Ph = C_6H_5.

High-performance liquid chromatography (HPLC) was performed on a Chromatem 380 instrument (Touzard and Matignon) with an SP 8200 single-wavelength (254 nm) detector. Separation was carried out on a 250 × 4.6 or a 150 × 4.6 mm I.D. stainless-steel column packed with 5-μm Hypersil SAS (C_1 or C_8) silica with a surface area of *ca.* 170 $m^2\ g^{-1}$. The precolumn was filled with pellicular silica. Water–*n*-propanol containing various amounts of the quaternary ammonium salt was used as the mobile phase, the flow-rate being 0.6 ml/min and the pressure about 10^3 p.s.i.

RESULTS AND DISCUSSION

In order to obtain some information on the conditions that allows the HPLC separation of the chiral sulphonated diphosphines, we made some preliminary studies on achiral sulphonated mono- and diphosphines and the corresponding oxides.

The influence of the cetrimide concentration on the capacity factor, k', of these achiral sulphonated phosphines and diphosphines **1**–**4**, and their oxides using water–*n*-propanol (5:2, v/v) as eluent is summarized in Table I. For the sulphonated phosphines derived from triphenylphosphine (**1a**–**c**) (entries 1–3), 1,2-bis(diphenylphosphino)ethane (**2a**–**d**) (entries 7–10) and 1,4-bis(diphenylphosphino)butane (**4a** and **b**) (entries 17 and 18), k' increased with increasing number of sulphonic groups in the molecule ($k'_{\mathbf{1a}} < k'_{\mathbf{1b}} < k'_{\mathbf{1c}}$, $k'_{\mathbf{2a}} < k'_{\mathbf{2b}} < k'_{\mathbf{2c}} < k'_{\mathbf{2d}}$ and $k'_{\mathbf{4a}} < k'_{\mathbf{4b}}$) for any concentration of cetrimide. For a given phosphine, a maximum k' was reached at a cetrimide concentration of about 6.8 m*M*. The same features were found for the corresponding oxides, the maximum k' being at a cetrimide concentration of about 14 m*M*. For the diphosphines having the same degree of sulphonation (**2c** and **4a**; **2d**, **3** and **4b**) and the corresponding phosphines oxides, k' increased with increasing hydrocarbon chain length between the two diphenylphosphino groups.

The sulphonated phosphines were generally eluted after the corresponding phosphine oxides. However, the reverse elution order was found for the tetrasulphonated diphosphines **2d**, **3** and **4b** for cetrimide concentrations up to 1.7 m*M*; the same behaviour was observed for the trisulphonated monophosphine **1c** at cetrimide concentrations up to 27 m*M*.

TABLE I

INFLUENCE OF CETRIMIDE CONCENTRATION ON THE CAPACITY FACTOR (k') IN THE SEPARATION OF THE SULPHONATED PHOSPHINES AND PHOSPHINES OXIDES **1–4**

Eluent, water–*n*-propanol (5:2); counter ion, cetrimide; column packing, 5-μm SAS (C_1) silica; flow-rate, 0.6 ml/min; pressure, 90 bar.

Entry No.	*Phosphine*	k'					
		[Cetrimide] (10^{-3} M)					
		1.69	*3.37*	*6.75*	*13.5*	*27*	*54*
1	**1a**	6.8	7.9	12.3	11.0	8.1	3.6
2	**1b**	7.5	10.6	21.0	17.6	9.6	4.5
3	**1c**	11.4	19.8	36.3	27.1	12.5	6.4
4	**1a** oxide	1.9	2.1	3.1	3.9	2.8	2.0
5	**1b** oxide	2.5	3.5	7.1	9.2	5.6	3.4
6	**1c** oxide	5.2	9.5	18.6	19.7	13.6	6.6
7	**2a**	4.3	6.8	11.0	11.0	6.8	3.9
8	**2b**	6.5	8.8	12.9	12.5	8.6	5.0
9	**2c**	11.1	14.0	18.9	18.1	10.9	6.6
10	**2d**	14.1	18.8	26.9	24.8	16.9	11.3
11	**2a** oxide	1.3	1.9	2.6	2.8	2.0	1.5
12	**2b** oxide	1.9	3.3	5.0	6.0	4.6	3.4
13	**2c** oxide	3.3	8.5	13.2	14.5	9.1	6.1
14	**2d** oxide	8.6	25.5	>40	>40	23.5	11.9
15	**3**	15.3	20.0	27.9	26.9	17.6	11.5
16	**3** oxide	9.1	27.8	>40.0	>40.0	24.0	12.0
17	**4a**	11.4	15.4	20.4	19.1	12.6	8.5
18	**4b**	16.0	21.6	30.9	28.9	18.8	12.0
19	**4a** oxide	3.5	9.3	13.5	14.8	11.0	7.8
20	**4b** oxide	9.3	29.5	>40.0	>40.0	25.3	12.8

TABLE II

INFLUENCE OF THE ALKYL CHAIN LENGTH OF THE TETRAALKYLAMMONIUM CATION ON THE CAPACITY FACTOR (k') IN THE SEPARATION OF SULPHONATED MONOPHOSPHINES **1**

Eluent, water–*n*-propanol (5:2); [counter ion], 0.027 *M*; column packing, 5-μm SAS (C_1) or 5-μm SAS (C_8) silica; flow-rate, 0.6 ml/min; pressure, 90 bar.

Column	*Counter ion*	k'		
		1a	***1b***	***1c***
C_1	TMA	0.86	0.10	0.02
	TEA	1.40	0.25	0.06
	TBA	3.6	1.6	1.0
	Cetrimide	8.1	9.6	12.5
C_8	TMA	1.90	0.12	0.01
	TEA	2.60	0.25	0.04
	TBA	4.20	0.88	0.46
	Cetrimide	7.80	10.90	16.50

The dependence of k' on the alkyl chain length of the counter ion was studied for the monophosphines **1a–c** using two columns (SAS C_1 and SAS C_8). The results in Table II show that the use of a counter ion with a shorter alkyl chain (tetrabutylammonium, tetraethylammonium or tetramethylammonium bromide) instead of cetrimide at the same mobile phase concentration reversed the elution order of the sulphonated phosphines, with **1c** now being eluted first and **1a** last. An increase in k' was also observed with increasing hydrophobicity of the counter ion, as reported previously[14,15].

We then studied the separation of chiral sulphonated diphosphines derived from (*S,S*)-1,2-bis[(diphenylphosphino)methyl]cyclobutane [(*S,S*)-cyclobutanediop], (*S,S*)-2,4-bis(diphenylphosphino)pentane [(*S,S*)-BDPP] and (*S,S*)-2,3-bis(diphenylphosphino)butane [(*S,S*)-Chiraphos]. The situation is more complex because the disulphonated and trisulphonated diphosphines are mixtures of two diastereoisomers, whereas the disulphonated diphosphines represent a mixture of three diastereoisomers. The results obtained with water–*n*-propanol (5:2) as the eluent and cetrimide (0.054 *M*) as the counter ion, which are summarized in Table III, show that for a given diphosphine or diphosphine oxide the retention time increases with increasing degree of sulphonation of the compound, following the behaviour of the achiral phosphines.

For (*S,S*)-BDPP, the monsulphonated diphosphine **6a** and the trisulphonated diphosphine **6c** gave two peaks in the ratio 50:50, corresponding, for each diphosphine, to the epimers at the phosphorus (*S,S,S*) and (*S,S,R*). The disulphonated (*S,S*)-BDPP **6b** exhibited three peaks in the ratio 25:50:25, corresponding to the three diastereoisomers at the phosphorus (*R,S,S,R*), (*R,S,S,S*) and (*S,S,S,S*). The same behaviour was found for the oxides of the sulphonated BDPP (Fig. 2).

For the (*S,S*)-cyclobutanediop, the oxide of the trisulphonated diphosphine **5b** also showed two peaks for the two epimers (*S,S,R*) and (*S,S,S*) at the phosphorus in the ratio 50:50, and the disulphonated diphosphine oxide **5a** three peaks for the three diastereoisomers at the two phosphorus (*R,S,S,R*), (*R,S,S,S*) and (*S,S,S,S*) in the

TABLE III

SEPARATION OF CHIRAL SULPHONATED PHOSPHINES AND THEIR OXIDES

Eluent, water–*n*-propanol (5:2); [cetrimide], 0.054 *M*; column packing, 5-μm SAS (C_1) silica; flow-rate, 0.6 ml/min; pressure, 90 bar.

Substrate	*k'*	
	Phosphine	*Phosphine oxide*
5a	5.4	3.5; 3.6; 4.3
5b	7.3	5.9; 6.5
5c	10.3	11.8
6a	3.3; 3.5	3.0; 3.4
6b	4.9; 5.2; 5.5	3.8; 4.2; 4.5
6c	7.2; 8.2	6.7; 7.6
6d	10.8	12.2
7a	5.2	3.4
7b	7.8	7.1
7c	11.8	13.8

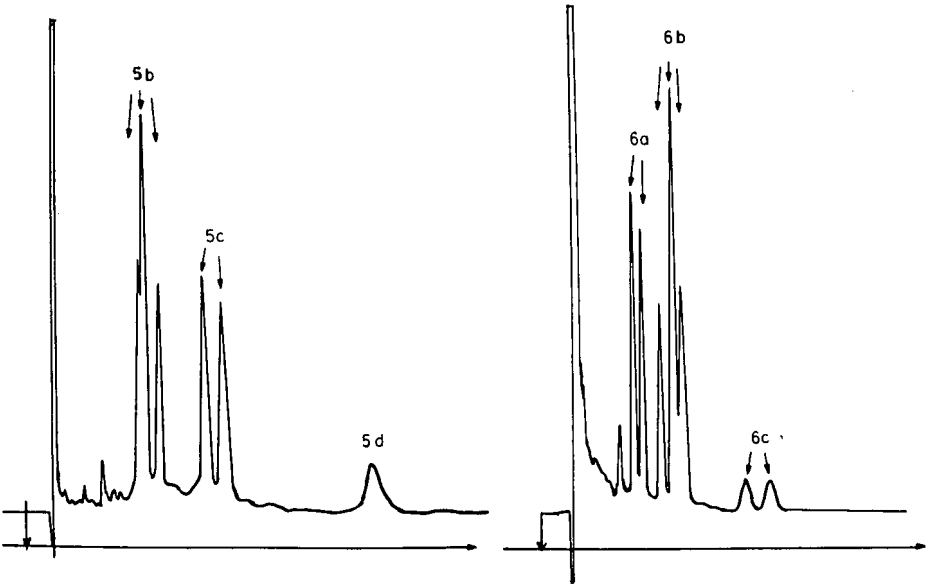

Fig. 2. Chromatogram of the di-, tri- and tetrasulphonated (*S,S*)-cyclobutanediop oxides (left) and of the mono-, di- and trisulphonated (*S,S*)-BDPP oxides (right). Eluent, water–*n*-propanol (5:2); [cetrimide], 0.054 *M*; column packing, 5-μm SAS (C_1) silica; flow-rate, 0.6 ml/min; pressure, 90 bar.

TABLE IV

INFLUENCE OF THE ALKYL CHAIN LENGTH OF THE TETRAALKYLAMMONIUM CATION ON THE CAPACITY FACTOR (*k'*) FOR MONO-, DI-, TRI- AND TETRASULPHONATED (*S,S*)-BDPP **6a–d**

Eluent, water–*n*-propanol (5:2) containing the counter ion; column packing, 5-μm SAS silica; flow-rate, 0.6 ml/min; pressure, 90 bar.

Column	*Ligand*	*k'*			
		Counter ion			
		TEA (0.027 M)	*TBA (0.027 M)*	*Cetrimide (0.027 M)*	*Cetrimide (0.054 M)*
C_1	**6a**: isomer 1	4.8	12.2	4.9	3.3
	isomer 2	3.7	9.1	5.2	3.5
	6b: isomer 1	0.9	6.6	7.9	4.9
	isomer 2	0.8	4.8	8.6	5.2
	isomer 3	0.6	4.0	9.3	5.5
	6c: isomer 1	0.3	2.9	12.0	7.2
	isomer 2	0.15	2.3	13.8	8.2
	6d	0.04	1.9	16.9	10.8
C_8	**6a**: isomer 1	4.4	23.3	9.6	4.6
	isomer 2	4.0	18.5	11.1	4.9
	6b: isomer 1	1.7	4.9	14.6	8.8
	isomer 2	1.2	3.8	16.5	9.6
	isomer 3	0.7	2.9	18.4	10.3
	6c: isomer 1	0.9	1.5	22.9	14.0
	isomer 2	0.09	1.0	24.9	16.5
	6d	0.02	0.6	30.5	20.8

ratio 25:50:25 (Fig. 2). However, the separation was not so good for the sulphonated (*S,S*)-cyclobutanediop **5a–c**.

For the sulphonated (*S,S*)-Chiraphos **7a–c** and their oxides, no separation of the diastereoisomers at the phosphorus was observed.

Using water–*n*-propanol (5:2) as the eluent, the influence of some parameters was studied in more detail for the sulphonated diphosphines **6a–d** derived from (*S,S*)-BDPP (Table IV). As found previously for the achiral diphosphines, k' increased when the cetrimide concentration decreased from 0.054 to 0.027 *M*. The use of counter ions with shorter alkyl chains, such as TEA and TBA, reversed the elution order of the sulphonated phosphines; for a given diphosphine, k' was higher with TBA than TEA.

CONCLUSION

Sulphonated diphosphines and their oxides were separated by ion-pair chromatography using an 5-μm Hypersil SAS C_1 column. The mobile phase was water–*n*-propanol (5:2) containing cetrimide (0.054 *M*); tetraethyl- or tetrabutylammonium bromide could also be used. The method allows a very easy and efficient determination of the composition of the mixtures obtained from the sulphonation of these phosphines, particularly for the chiral diphosphines used in asymmetric catalysis.

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PUBLICATION SCHEDULE FOR 1990

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

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